

## REMARKS

In general, the present invention provides methods of treating, reducing, or preventing pain by administering to a mammal in need thereof a nucleic acid encoding a constitutively active mu opioid receptor in an effective amount.

### Sequence Listing

Applicants enclose herewith a corrected sequence listing (paper copy and computer readable format) in accordance with 37 C.F.R. § 1.821 through § 1.825 along with a sequence statement. As required by the Examiner, the sequences are now consistent with the text of the specification. Support for these amendments is found, for example, in FIGURES 1, 13, and 14. No new matter has been added by these amendments.

### The Office Action

Claims 1-33 are pending. Claims 1-33 are rejected under 35 U.S.C. § 112, first paragraph. Claims 1 and 3 stand rejected under 35 U.S.C. § 112, second paragraph. Each of these rejections is discussed in detail below.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-33 stand rejected under 35 U.S.C. § 112, first paragraph for lack of enablement based on three asserted grounds. First, the Office states that “the specification fails to provide a correlation to therapeutic levels of expression of a nucleic acid encoding a constitutively active or hypersensitive mu opioid receptor in an *in vivo* setting in any subject having pain.” Second, citing various references such as Verma *et al.* (*Nature* (1997) 389: 239-242; hereinafter ‘Verma’), Eck *et al.* (*Phar. Basis Ther.* (1996): 77-101), Miller *et al.* (*FASEB J.* (1995) 9:190-199), and Iadarola *et al.* (*Molecular Neurobiology of Pain* (1997) 9:337-359), the Office states that gene therapy generally is unpredictable and concludes that claims 1-33 are therefore not enabled. And third, citing Wang *et al.* (*Life Sciences* (1994) 54:339-350) and Sadee *et al.* (*Analgesia* (1994) 1:11-14) for the proposition that opioid dependence may result from Applicants’ therapeutic approach, the Office states that there is a question as to “whether adding a constitutively active mu-opioid receptor via gene therapy would result in adverse effects including drug tolerance and withdrawal.” For the reasons outlined below, each of these bases for the rejection are respectfully traversed.

Turning first to the Office’s assertion that *in vivo* experimental data must be presented in Applicants’ specification, Applicants note that the case law does not support such a requirement. In this regard, the M.P.E.P. clearly states (emphasis added):

*An applicant need not have actually reduced the invention to practice prior to filing. In Gould c. Quigg, 822 F.2d 1074, 1078, 3 USPQ 2d 1302, 1304 (Fed. Cir. 1987), as of Gould’s filing date, no person had built a light amplifier or measured a population inversion in a gas discharge. The Court held that “The mere fact that something has*

not previously been done clearly is not, in itself, a sufficient basis for rejecting all applications purporting to disclose how to do it.” 822 F.2d at 1078, 3 USPQ2d at 1304 (quoting *In re Chilowsky*, 229 F.2d 457, 461, 108 USPQ 321, 325 (CCPA) 1956)).

Rather, the case law is clear that “[T]he specification need not contain an example if the invention is otherwise disclosed in such a manner that one skilled in the art will be able to practice it without undue experimentation. *In re Borkowski*, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).” Because Applicants’ specification provides extensive enabling details concerning the administration of a gene encoding a constitutively active mu-opioid receptor for the purpose of treating, reducing, or treating pain in a mammal, Applicants submit that, in spite of the lack of *in vivo* working examples, the present specification is in compliance with the enablement requirement of 35 U.S.C. § 112, first paragraph.

In particular, claim 1, from which claims 2-33 depend, is directed to a method of treating, reducing, or preventing pain in a mammal by administering a nucleic acid encoding a constitutively active mu-opioid receptor. The Office asserts that the specification fails to teach “how to construct an effective therapeutic viral or non-viral vector, which cells types are effectively targeted by which vector, how to deliver a given vector such that it reaches targeted by which vector, how to deliver a given vector such that it reaches targeted cells, or that any therapeutic level of expression could be achieved to effect a therapeutic response to any particular type of pain, in any particular tissue,” Applicants respectfully disagree and point out that, at the time of filing, based on the

teachings of the specification, one skilled in the art could have readily practiced the claimed invention without undue experimentation.

On this point, Applicants direct the Examiner's attention to the specification where various conditions that are amenable to treatment according to the methods of the present invention are listed at page 43, lines 12-21, including, for example, chronic back pain resulting from any etiology (e.g., fractures or metastatic diseases). The specification further provides ample guidance concerning the construction of nucleic acids encoding constitutively active mu-opioid receptors. Exemplary constitutively active mu-opioid receptors that may be employed according to the present invention are described at page 44, lines 1-18. These receptors may be engineered into viral or non-viral vectors for administration to a mammal. Exemplary viral vectors include adenoviruses, retroviruses, adeno-associated adenoviruses, lentiviruses, papova virus-based vectors, herpes virus-based vectors, viral vectors that contain or display the vesicular stomatitis virus G-glycoprotein spike, Semi-Forest virus-based vectors, Hepadnavirus-based vectors, and bavulovirus-based viruses (see, for example, page 3, lines 18-19, page 43, lines 3-11, page 45, line 5, page 48, line 11). With respect to non-viral vectors, various methods useful for their delivery are also taught in the present application and include, for example, microinjection, calcium phosphate transfer, lipofection, asialorosonucoid-polylysine conjugation, electroporation, liposome, receptor-mediated endocytosis of DNA (see page 48, line 13, through page 50, line 1), and administration by an accelerated particle gene transfer gun (see page 51, line 11, through page 52, line 3). Although the

use of viral and non-viral vectors generally leads to protein expression in a vast spectrum of cell types, the specification discloses various methods that would provide effective, targeted delivery of the mu-opioid receptor nucleic acid to particular cell types. The specification teaches, for example, that retroviral vectors characterized by specific neural tropisms may be particularly useful. The therapeutic nucleic acids of the invention may also be delivered to a desired cell type using, for example, cell-specific ligands that target administration to a specific cell in the mammal being treated (see, for example, page 3, lines 19-20 and page 24, line 17, through page 25, line 15). Alternatively, tissue-specific promoters may also drive the expression of the constitutively active mu receptor in a specific cell type.

The present specification further teaches that the mu-opioid receptor nucleic acids may be administered to the mammal being treated using a variety of routes, including administration intravenously, intramuscularly, intraperitoneally, subcutaneously, nasally, orally, by suppository, by transdermal patch, or by inhalation (see page 52, lines 4-9). The specification discloses that the nucleic acid is preferably administered to a site at which a therapeutic benefit is desired, including surfaces (e.g., skin or mucosal surfaces such as bronchial/nasal passages or the genitourinary tract) (see page 22, lines 2-9). For example, the specification specifically teaches that an adenoviral vector encoding the constitutively active mu-opioid receptor may be injected, for example, into the intrathecal space (see page 43, lines 7-11), or to target a specific organ, for example, the nucleic acid may be directly administered by injection to that organ (see page 21, lines 19-20).

With respect to achieving therapeutic levels of expression, Applicants note that dosage determination may be carried out routinely, using methods known as of the filing date. For example, the vectors encoding constitutively active mu-opioid receptors may be analyzed in order to determine both the levels of transgene product generated as well as the activity of the mu-opioid receptor produced, for example, *in vivo*. Based on such characterization, one skilled in the art may routinely determine an appropriate number of DNA molecules to administer using standard methods. Dosage may therefore be adjusted by, for example, administering more or less vector DNA in order to reach a known, therapeutically acceptable amount. In this particular respect, the specification states: “One skilled in the art will appreciate that the therapeutic composition is preferably administered at a unit dose sufficient to reduce or eliminate the symptoms of a disease or disorder in a mammal, and such a dose can be easily determined by one of ordinary skill in the art” (see page 4, lines 5-8). The activity of the constitutively active receptor of the invention may further be titrated to increase or decrease the level of receptor signaling (see page 25, lines 16-20). The specification further teaches that expression levels may be controlled by means of constitutive promoters, inducible promoters, or tissue-specific promoters (see, for example, page 26, lines 1-12). Exemplary quantities as well as suitable dose regimes are provided at page 52, line 10, through page 53, line 19.

Furthermore, in terms of achieving high levels of expression, it was well known in the art at the time of filing that the use of adenoviruses in particular could result in both high expression of the protein product and a large number of cells being infected due to

the ability of the virus to infect non-proliferating cells. Despite its transient nature, such expression may be sufficient to treat or reduce pain and may even be advantageous in light of possible bio-safety concerns associated with long-term expression of a constitutively active mu-opioid receptor gene. Because the claims are not limited to single-dose administration, repeated, even indefinite, administration of a vector encoding a constitutively active mu-opioid receptor can be used to prevent and treat certain cases. Furthermore, it was well known in the art at the time of filing that the use of various improved techniques could also result in both sustained transgene expression lasting for months in most systems. Such techniques involve, for example, the use of “second generation” vectors or alternatively, the use of recombinant adeno-associated viruses (rAAV), which are taught in the present specification. Even the cited Verma reference states that long-term transgene expression can be achieved if the recombinant adenoviral vector is administered concomitantly with immunosuppressive agents or using second-generation ‘gut-less’ vectors, which can escape the immune system (page 241).

In view of the above, Applicants submit that the present specification enables the currently claimed methods and provides guidance to those skilled in the art on how to carry out each and every step mentioned by the Office in making the § 112 rejection. This basis for the rejection should be withdrawn.

Similarly, the Office’s reliance on the general state of gene therapy is also an inappropriate basis for concluding that Applicants’ methods are not enabled. To emphasize the unpredictability of gene therapy strategies, the Examiner cites Verma, in

which it is stated that “out of the more than 200 clinical trials currently underway, no single outcome can be pointed to as a success story.” Citing various additional references, the Examiner attributes this lack of success to the various obstacles faced by gene therapy strategies, including the choice of DNA vector and the intricate regulation of gene expression in cells. Applicants respectfully disagree with the Examiner’s position on the lack of success of gene therapy strategies and submit that the general treatment of pain in *in vivo* models using gene therapy strategies is in fact predictable. Applicants note that a number of studies using gene therapy strategies in animal models, including those generally taught in Applicants’ specification, had been performed successfully at or around the time of filing of the application; some were even performed for the explicit purpose of treating pain.

As evidence of this assertion, Applicants submit Exhibits A-D. In Exhibit A, Finegold *et al.* (*Human Gene Therapy* (1999) 10:1251-1257) successfully demonstrated a marked reduction of chronic pain by a specific opioid receptor-mediated mechanism. In these experiments, administration of an adenoviral vector encoding a secreted form of the potent endogenous opioid  $\beta$  endorphin into the cerebrospinal fluid surrounding the spinal chord resulted in a marked reduction in inflammation-mediated hyperalgesia, a form of pain. The fact that gene intervention could be achieved at the level of the spinal chord indicates that pain resulting from a broad spectrum of disorders may be treated in a similar manner. Similarly, in Exhibit B, Braz *et al.* (*J. Neuroscience* (2001) 21:7881-7888) demonstrates that a recombinant herpes simplex virus encoding the rat



proenkephalin A-precursor protein, a precursor of opioid peptide involved in the control of pain, could be used to enhance enkephalin synthesis in sensory neurons of polyarthritic rats, thereby improving locomotion concomitant with a reduction in hyperalgesia. And Goss *et al.* (*Gene Therapy* (2001) 8:551-556) (EXHIBIT C) demonstrated that transfection of neurons of the dorsal root ganglion *in vivo* with a herpes simplex viral vector encoding a proenkephalin gene could successfully elicit a pronounced anti-nociceptive effect (nociception being the sensation of pain). Moreover, in these experiments, while the antinociceptive effect diminished after four weeks, re-inoculation of the vector re-established the analgesic effect in the absence of any evidence of tolerance. Wilson *et al.* (*Proc. Natl. Acad. Sci. USA* (1999) 96:3211-3216) (EXHIBIT D) also provides evidence that pain reduction may be induced by gene therapy. In Wilson, a recombinant herpes simplex viral vector encoding proenkephalin, an opioid peptide precursor, was applied to the abraded skin of the dorsal hindpaw of mice, and shown to markedly reduce or eliminate the sensitization of the foot withdrawal response following the application of capsaicin or DMSO. This response was observed as soon as day 4-5 post-infection and remained robust for at least seven weeks. In view of these studies, Applicants submit that there can be no question that a person of ordinary skill in the art could have practiced the present invention without undue experimentation using the teachings in the specification and standard molecular biology techniques. Moreover, given the success achieved with other gene therapy approaches for reducing pain, Applicants submit that in this field there is a high level of predictability for a positive

therapeutic outcome. This second basis for the rejection should also be withdrawn.

The third and final basis for the enablement rejection focuses on the citation of Wang *et al.* (*Life Sciences* (1994) 54:339-350) and Sadee *et al.* (*Analgesia* (1994) 1:11-14) for the proposition that constitutive mu-opioid receptor activation may lead to increased opioid dependence. The Examiner raises the question of “whether adding a constitutively active mu-opioid receptor via gene therapy would result in adverse effects including drug tolerance and withdrawal” and concludes that the present disclosure is not enabling simply because “it cannot be predicted how long and how much constitutively active mu opioid receptor would be present in a cell if administered by gene therapy.” This basis for the rejection should also be withdrawn. The fact that the present technique may have side effects is not germane to the issue of enablement. Indeed, most approaches to pain reduction entail some amount of drug tolerance, dependency, or withdrawal. Rather, all that is relevant is whether Applicants’ specification teaches one skilled in the art how to carry out the claimed invention without undue experimentation. As indicated above, Applicants have satisfied this requirement of § 112, first paragraph.

The § 112, first paragraph rejection should be withdrawn.

#### Rejection under 35 U.S.C. § 112, second paragraph

Claims 1 and 3 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite.

The Office asserts that the term “sufficient” in claim 1 is unclear given that the

phrase “disease-inhibiting amount” is vague and unclear and does not adequately describe what an “amount sufficient to treat” is. Applicants respectfully disagree and submit that one skilled in the art reading the specification would clearly understand that by the term “sufficient” is meant the amount necessary to treat, reduce, or prevent pain in the mammal being treated. According to the definition of the phrase “disease-inhibiting amount” or “disorder-inhibiting amount” found at page 11, lines 7-14, this amount is the amount of nucleic acid encoding a constitutively active mu-opioid receptor that when delivered to a cell, tissue, or site is capable of reducing, delaying or stabilizing pain in a mammal. Applicants note that one of skill in the art may readily determine such an amount using standard approaches in the art, and accordingly this aspect of the § 112, second paragraph rejection should be withdrawn.

The Examiner also finds the phrase “human equivalent” in claim 3 indefinite because it is unclear whether the claim is referring to a human equivalent of the specific mutation or a human equivalent of SEQ ID NO: 1. Claim 1 has been amended to specify that the “human equivalent” of the rat mu-opioid receptor corresponds to SEQ ID NO: 78. This aspect of the rejection may be withdrawn.

## CONCLUSION

Applicants submit that this case is now in condition for allowance, and such action is respectfully requested.

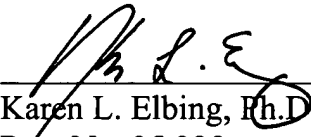
Applicants draw the Examiner's attention to the Information Disclosure Statement that was submitted after the mailing of the first Office Action on June 16, 2003, and request that this Statement be reviewed and the Form PTO-1449 accompanying that Statement be initialed and returned with the next action.

Enclosed is a Petition to extend the period for replying to the Office action for three months, to and including October 17, 2003, and a check in payment of the required extension fee.

If there are any charges or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 16 October 2003

  
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HUMAN GENE THERAPY 10:1251-1257 (May 1, 1999)  
Mary Ann Liebert, Inc.

## A Paracrine Paradigm for *in Vivo* Gene Therapy in the Central Nervous System: Treatment of Chronic Pain

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### ABSTRACT

A limitation of current gene therapy efforts aimed at central nervous system disorders concerns distribution of vectors on direct injection into neural tissue. Here we have circumvented this problem by transferring genes to the meninges surrounding the spinal cord, achieving an *in vivo* gene transfer paradigm for treating chronic pain. The therapeutic vector consisted of a recombinant adenovirus encoding a secreted form of the potent endogenous opioid  $\beta$ -endorphin. In an inflammation model of persistent pain, administration of the vector into the cerebrospinal fluid (CSF) surrounding the spinal cord transduced meningeal pia mater cells. The resulting increase in  $\beta$ -endorphin secretion attenuated inflammatory hyperalgesia, yet had no effect on basal nociceptive responses. This demonstration of a gene transfer approach to pain treatment can be generalized to neurodegenerative disorders in which broad spatial distribution of therapeutic effect is critical.

### OVERVIEW SUMMARY

One of the underappreciated aspects that limits viral gene therapy in the CNS concerns inadequate tissue distribution of viral vectors. A novel approach is presented, in which a therapeutic product is delivered according to a meningeal-paracrine paradigm. As a test case, adenoviral delivery of a  $\beta$ -endorphin gene to meningeal cells surrounding the spinal cord specifically reduced chronic pain behavior in a rat model. In terms of pain control at the spinal level, this approach to pain gene therapy is easily applied as an outpatient therapy by lumbar puncture. The clinical utility of this meningeal-paracrine approach is broadly applicable to other CNS disorders in which large areas of brain tissue and/or large numbers of distributed neurons must be affected, such as Parkinson's or Alzheimer's disease, or amyotrophic lateral sclerosis.

### INTRODUCTION

CONTROL OF CHRONIC PAIN is a worldwide public health problem (Bonica, 1990; Wall and Melzack 1994; Gureje *et al.*, 1998). Many disease processes and traumatic injuries can lead

to a lifetime of unremitting severe pain. Current treatments often fall short of therapeutic goals and can eventually involve procedures that are invasive or associated with unacceptable side effects. In neuropathic pain after nerve injury, pain is poorly controlled by currently available methods. In cancer pain, intravenous or oral morphine is only partially effective, and is accompanied by debilitating side effects such as constipation, sedation, and respiratory depression. Furthermore, its practical use is often limited by concerns about addictive properties (Bonica, 1990; Wall and Melzack, 1994; American Pain Society, 1995; Bernabei *et al.*, 1998; Gureje *et al.*, 1998; Ingham and Foley, 1998). Attempts to localize delivery of opiates in order to reduce side effects include both mechanical and cellular approaches. Catheters can be implanted into the subarachnoid space and connected to infusion pumps, but are costly, difficult to maintain, and present an infection risk. Spinal grafts of clonal cell lines (Wu *et al.*, 1994; Beutler *et al.*, 1995) or xenografts of primary tissue (Sagen, 1998) have also been proposed, but are constrained by issues of control, maintenance, and invasive surgery.

One way to circumvent such issues and provide long-term pain control is to directly modulate pain through *in vivo* gene transfer (Iadarola *et al.*, 1997; Jacoby *et al.*, 1997; Anderson, 1998). In this article we focus on gene transfer intervention in

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the spinal cord, because pain from a broad spectrum of disorders can be controlled at this level of the central nervous system. Previous studies on direct intraparenchymal injections of adenoviral vectors (Ad-lacZ) expressing a lacZ transgene resulted in nonuniform distribution of transgene expression and limited spread of expression in neural tissue (Mannes *et al.*, 1998). This is a general problem in gene delivery to the central nervous system, and in particular is a problem in pain treatment. Since primary afferent nociceptive (pain) neurons from a given region of peripheral tissue innervate several spinal segments, multiple injections are required to achieve a significant anti-pain effect.

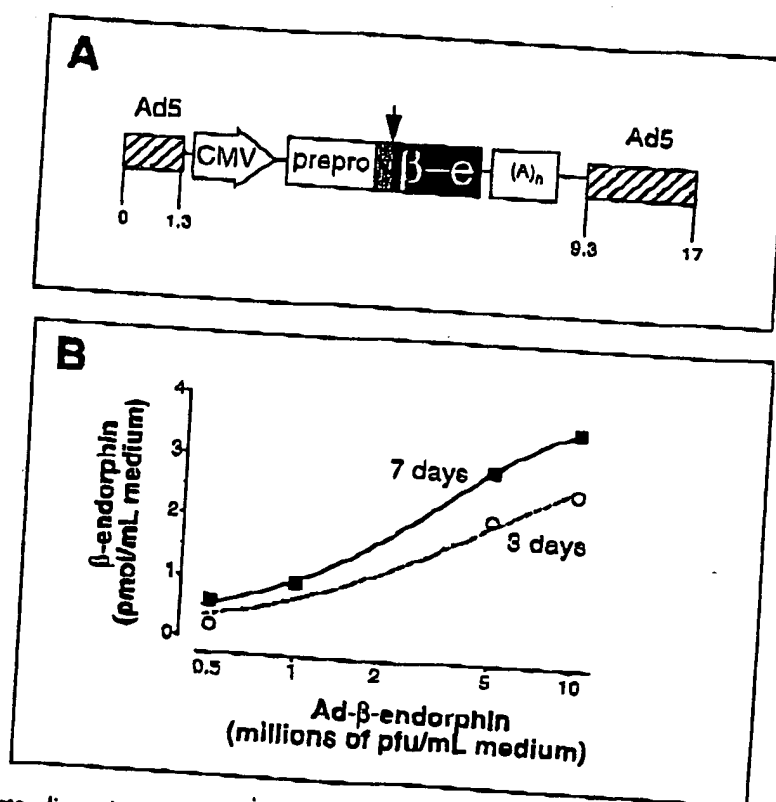
In contrast to intraspinal injections, intrathecal delivery of Ad-lacZ into the cerebrospinal fluid (CSF) surrounding the spinal cord did not yield viral access to cells of the spinal cord itself, but rather to cells of the pia mater, the meningeal layer directly apposed to the spinal cord (Mannes *et al.*, 1998). The pia mater thus provided a highly accessible target tissue for gene transfer using a meningeal-paracrine secretory approach.  $\beta$ -Endorphin is a neuropeptide with known pain-suppressing properties in humans and animals (Yaksh and Henry, 1978; Oyama *et al.*, 1980; Max *et al.*, 1985). Secretion of  $\beta$ -endor-

phin peptide from nonneural cells is efficiently directed by addition of a heterologous prepro-sequence from the nerve growth factor gene (Beutler *et al.*, 1995). The approach in the present report integrated these three sets of observations for therapeutic delivery of  $\beta$ -endorphin to the spinal cord via the pia mater. Given the ease of access to the pia mater, our strategy for functional intervention was to control neuronal excitability by local secretion of a neuromodulatory peptide,  $\beta$ -endorphin.

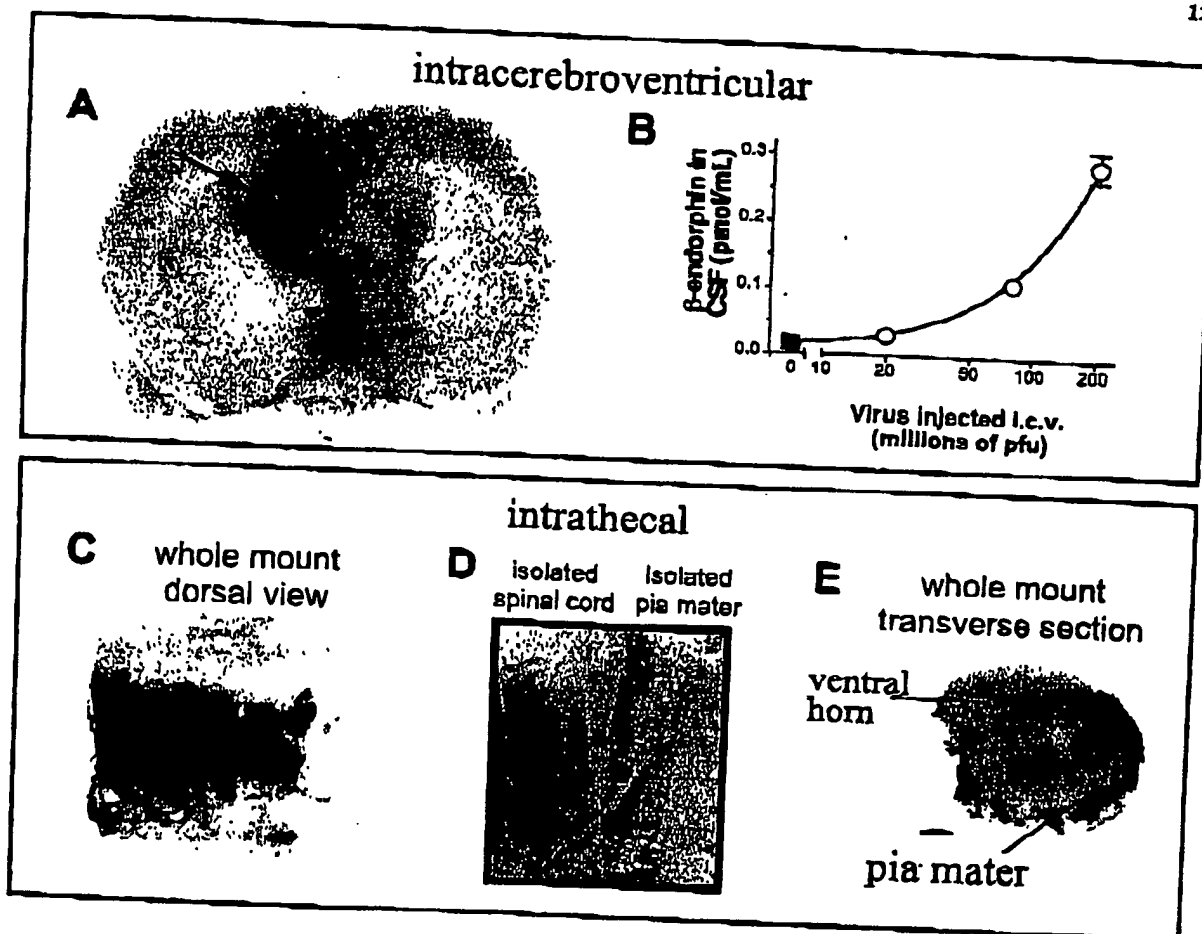
## MATERIALS AND METHODS

### Vector construction

A fusion gene (Beutler *et al.*, 1995) consisting of the nerve growth factor prepro-sequence followed by the  $\beta$ -endorphin peptide was ligated into the *EcoRI*-*Bam*HI restriction sites of pACCMV-pLpA (Becker *et al.*, 1994). The resulting recombinant adenoviral vector (first-generation, replication-deficient Ad5) containing the expression cassette was concentrated and purified. The titer of the final viral preparation was determined by absorption spectroscopy for particle number ( $3 \times 10^{11}/\text{ml}$ ).



**FIG. 1.** Adenoviral vector-directed expression of secreted  $\beta$ -endorphin from cells cultured *in vitro*. (A) Structure of Ad- $\beta$ -endorphin vector. An Ad5 recombinant (adenovirus sequences are hatched, map units indicated) expresses a fusion between a nerve growth factor prepro-sequence and  $\beta$ -endorphin ( $\beta$ -e), under control of the cytomegalovirus promoter (open arrow) and polyadenylation sequences ( $A$ )<sub>n</sub>. Sequences (gray) directing cleavage by prohormone convertases (arrow) bridge the fusion gene. (B) Production of  $\beta$ -endorphin by bovine smooth muscle cells transduced by Ad- $\beta$ -endorphin. Medium from cells incubated with indicated concentrations of virus was assayed for  $\beta$ -endorphin by radioimmunoassay.  $\beta$ -Endorphin levels at 3 days (open circles, dotted line) and 7 days (filled squares, solid line) postinoculation are plotted and superimposed by curves fitted to hyperbolae.



**FIG. 2.** *In vivo* gene transfer and production of  $\beta$ -endorphin. (A) X-Gal histochemistry of a coronal section of a rat brain 2 days after injection of Ad-lacZ and Ad- $\beta$ -endorphin into the lateral ventricle. Note the blue product in ependymal cells of the lateral (arrow) and third (arrowhead) cerebral ventricles. Some staining of the needle track is evident in the cortex. (B) Dose response of Ad- $\beta$ -endorphin *in vivo* (open circles). Various amounts of Ad- $\beta$ -endorphin injected intracerebroventricularly (i.c.v.) directed secretion of  $\beta$ -endorphin, which was measured in CSF collected 2 days postinjection. Levels of  $\beta$ -endorphin in CSF from naive rats are indicated by a filled square. (C-E) Intrathecal administration of Ad-lacZ. At 3 days postinjection, spinal cords stained with X-Gal were viewed in three ways: (C) whole-mount view of dorsal aspect of spinal cord with intact pia mater; (D) after separation of the pia (blue stain, right) from the spinal cord itself (no stain, left); and (E) transverse section, tilted to visualize both the blue pia expression and the lack of stain in the parenchyma of the spinal cord. See (C) for  $\beta$ -endorphin secretion into CSF after intrathecal injection of Ad- $\beta$ -endorphin.

or infection of HEK293 cells ( $4 \times 10^9$  plaque-forming units [PFU]/ml).

#### Cell culture

Bovine smooth muscle cells (AG08595A; Coriell Institute, Camden, NJ) were grown to confluence ( $3.8 \times 10^6$  cells per well) in 11-mm dishes, with daily changes of medium. Various concentrations of Ad- $\beta$ -endorphin were added to wells and the medium was sampled on days 3 and 7 postinoculation.

#### $\beta$ -Endorphin radioimmunoassay

$\beta$ -Endorphin levels in the cell culture medium and rat CSF were determined by radioimmunoassay (Nichols Institute Diagnostics, San Juan Capistrano, CA). For sampling of rat CSF,

a 27-gauge hypodermic needle attached to a syringe with no hub or a clear hub (essential for visualization of CSF withdrawn) was inserted into the cisterna magna and approximately 100  $\mu$ l of CSF was removed. CSF or cell culture medium was boiled in water, clarified by centrifugation ( $12,000 \times g$ , 5 min), and stored frozen at  $-70^\circ\text{C}$  until assay.

#### Histological methods

After perfusion with 4% paraformaldehyde (but no postperfusion fixation), reaction of 2-mm-thick coronal slices of brains or sections of spinal cord with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal, pH 7.3, 1 mg/ml at  $37^\circ\text{C}$  (Shimohama *et al.*, 1989) demonstrated a blue reaction product within 15 min, which was complete by 2 hr.

*In vivo methods*

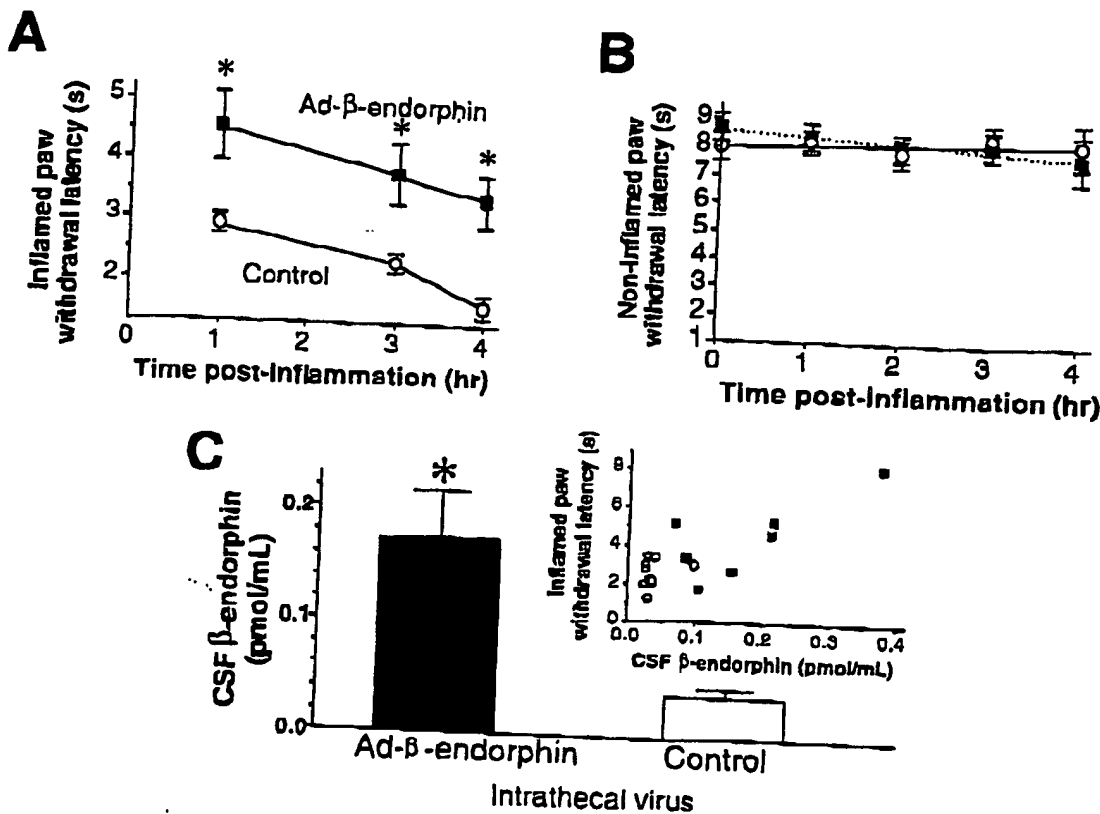
Animal experiments were performed in accordance with National Institutes of Health Animal Care and Use Committee guidelines. For intracerebroventricular injections, virus stock solution was diluted into 50 mM Tris (pH 7.4), 10% glycerol, 10 mM  $MgCl_2$  and infused into the left lateral cerebral ventricle (10  $\mu$ l over 10 min). The same viral solution was infused intrathecally (10  $\mu$ l over 2 min) through a PE-10 catheter. The catheter was inserted through an incision in the cisterna magna and advanced 3 cm, to approximate the rostral extent of the spinal cord segments innervated by the hindpaw (the peripheral site tested for nociceptive responses). Inflammation was induced by subcutaneous injection of 2 mg of carrageenan (type IV, C-3889; Sigma, St. Louis, MO) in a volume of 0.15 ml of phosphate-buffered saline (PBS) into the plantar surface of one hindpaw (200 to 300-g male Sprague-Dawley rats). After inflammation, there was no difference in edema (paw thickness) between Ad- $\beta$ -endorphin rats and controls (both increased by

approximately twofold). A radiant thermal test in unrestrained rats (Hargreaves *et al.*, 1988) was used to assess nociceptive responses by paw withdrawal latency. The observer was blinded as to the virus (control or Ad- $\beta$ -endorphin) injected.

## RESULTS

*Secretion of  $\beta$ -endorphin in vitro and in vivo*

Transgene expression from a recombinant adenoviral vector, named Ad- $\beta$ -endorphin (Fig. 1A), was controlled by a constitutive viral promoter. Secretion of  $\beta$ -endorphin from cells cultured *in vivo* with Ad- $\beta$ -endorphin increased with time and vector concentration from a minimum of 0.004 pmol/ml medium (0.5 million PPU/ml medium at 3 days) to a maximum of 3.5 pmol/ml medium per day at 7 days postinoculation (Fig. 1B), suggesting that such secretion might be sufficient to affect neuronal function *in vivo*.



**FIG. 3.** Nociceptive behavior of rats in a carrageenan model of inflammatory pain. Three days prior to the pain test, rats were infused intrathecally over 2 min with  $10^{10}$  particles of Ad- $\beta$ -endorphin (filled squares) or Ad-lacZ (open circles), in a volume of 10  $\mu$ l. (A) Attenuation of hyperalgesia by Ad- $\beta$ -endorphin. Peripheral inflammation (Hargreaves *et al.*, 1988) induced hyperalgesia in one hind paw of rats injected intrathecally with Ad-lacZ. A significant prolongation of latencies in Ad- $\beta$ -endorphin-treated rats was observed (data pooled from 12–27 animals,  $p < 0.005$ , two-tailed  $t$  test, indicated by asterisks) during the inflammation test period. (B) Noninflamed paw withdrawal latency after injection of Ad- $\beta$ -endorphin. No significant difference between control and Ad- $\beta$ -endorphin groups was observed (linear regression analysis). (C)  $\beta$ -Endorphin measurements from cisternal CSF of animals injected intrathecally with control or Ad- $\beta$ -endorphin viruses. CSF was drawn 7 days postinjection to allow cisternal wound healing. *Insert:* A trend ( $p < 0.08$ ) between increased paw withdrawal latencies and  $\beta$ -endorphin was observed after intrathecal administration of Ad- $\beta$ -endorphin.



## CNS THERAPY FOR CHRONIC PAIN

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Introduction of the virus into the lateral ventricle of rats (intracerebroventricular injection) allowed determination of the ability of the virus to secrete  $\beta$ -endorphin after *in vivo* gene transfer to ependymal cells lining the cerebral ventricles. The extent of gene transfer to ependymal cells was visualized by coinjection of Ad-lacZ (Becker *et al.*, 1994) and detection of the transgene product by X-Gal histochemistry (Fig. 2A) and immunocytochemistry (not shown). Both techniques demonstrated cells strongly positive for the transgene product in the ependymal layer. This layer formed a barrier to virus penetration into brain, since little or no expression occurred in neurons or glia beyond the ependymal layer (Fig. 2A; noted also in Bajocchi *et al.*, 1993). Injection of Ad- $\beta$ -endorphin produced a rapid, vector dose-dependent increase in CSF  $\beta$ -endorphin. As soon as 24 hr postinjection, the amount of  $\beta$ -endorphin secreted into the CSF reached 0.3 pmol/ml with 200 million PFU of virus, the maximum amount injected (Fig. 2B). Three days after intracerebroventricular injection, the level increased to 0.6 pmol/ml. These levels represent a 10- to 20-fold increase over normal  $\beta$ -endorphin levels in human or rat CSF, 0.01–0.03 pmol/ml (Kiser *et al.*, 1983; Nakao *et al.*, 1980).

Intrathecal injections rostral to the lumbar spinal cord were used to assess viral spread and transgene expression. Three days after intrathecal Ad-lacZ injection, the spinal cord was removed with pia mater attached and stained for transgene expression. Expression was observed in cells of the pia mater (Fig. 2C–E) but not of the spinal cord itself (Fig. 2D and E), evident after removal of the pia mater (Fig. 2D) or transverse section (Fig. 2E). The expression spread 2–3 cm in the rostral–caudal dimension. In animals injected intrathecally with Ad- $\beta$ -endorphin, CSF  $\beta$ -endorphin content, sampled from the cisterna magna, was 0.2 pmol/ml at 7 days and returned to endogenous levels (0.03 pmol/ml) by 15 days. The cisterna magna is anterior to the catheter tip and may not be the optimal site for sampling secretion from spinal pia mater, and thus these data may represent an underestimate of  $\beta$ -endorphin secretion. Data also suggest that transgene expression peaked between 3 and 7 days and waned after 15 days, a time course similar to that observed by other investigators (Bajocchi *et al.*, 1993; Monneé *et al.*, 1998). These data demonstrate secretion from both ependymal and pia mater cells.

### Effect of $\beta$ -endorphin on pain in rats

On the basis of these data, the ability of Ad- $\beta$ -endorphin to attenuate nociceptive (pain) responses in the peripheral inflammation model was examined following gene transfer to the spinal pia mater. To control for nonspecific effects of the viral vector, Ad-lacZ was injected intrathecally. Baseline thermal nociceptive tests in unrestrained rats prior to inflammation indicated that paw withdrawal latencies to a radiant thermal stimulus were  $8.7 \pm 2.6$  sec for Ad-lacZ, and  $8.9 \pm 2.5$  sec for Ad- $\beta$ -endorphin (mean  $\pm$  standard error;  $N = 33$  for each virus). In the control group, inflammation of one hind paw produced a marked decrease in withdrawal latency (e.g., to  $2.3 \pm 0.7$  sec at 3 hr postinflammation), an indication of hyperalgesia. Injection of Ad- $\beta$ -endorphin significantly attenuated the hyperalgesic response at each time point tested (1, 3, and 4 hr; Fig. 3A). Because the primary afferent inputs are lateralized, the inflammation affects only one side of the rat and the con-

tralateral, noninflamed, paw of these animals serves as an internal control for nonspecific or generalized effects on nociceptive responses. Latencies of this control paw remained constant during the period of behavioral testing for both control (Ad-lacZ) and Ad- $\beta$ -endorphin groups (Fig. 3B). This indicated a lack of toxicity or nonspecific pharmacological effects from either virus, emphasizing that the effect of  $\beta$ -endorphin was observed only during neural activation by inflammation of the primary afferent.

Animals injected with Ad- $\beta$ -endorphin exhibited significantly higher levels of  $\beta$ -endorphin than did control Ad-lacZ animals (Fig. 3C;  $0.17 \pm 0.04$  versus  $0.037 \pm 0.007$  pmol/ml;  $p < 0.007$  by *t* test). Although we were unable to obtain CSF from all animals tested, a trend in the degree of antihyperalgesia (measured 3 hr postinflammation) and increased  $\beta$ -endorphin levels was apparent in the Ad- $\beta$ -endorphin group (Fig. 3C, inset;  $r^2 = 0.42$ ,  $p < 0.08$ ). These data provide further support for the hypothesis that secretion of the transgene product specifically affected hyperalgesic responses seen with persistent pain.

Administration of naloxone, a specific opioid antagonist, tested the hypothesis that the antihyperalgesic actions of Ad- $\beta$ -endorphin were due to an opioid mechanism. For this experiment, two groups of 18 animals, one injected intrathecally with Ad-lacZ and the other with Ad- $\beta$ -endorphin, were inflamed as in Fig. 3A with carrageenan for 3 hr. Thermal testing of these animals confirmed that the Ad- $\beta$ -endorphin group was less hyperalgesic than the control group (Fig. 4). At this point, naloxone was delivered systemically. Determination of paw withdrawal latencies 40 min after naloxone revealed that the antihyperalgesic effect of Ad- $\beta$ -endorphin was reversed ( $p < 0.04$ , one-tailed *t* test; Fig. 4). Thus, an opioid receptor mechanism attenuated the antihyperalgesia due to Ad- $\beta$ -endorphin.

## DISCUSSION

The method of gene transfer to nonneural cells to affect neuronal function, described here, is a highly accessible para-

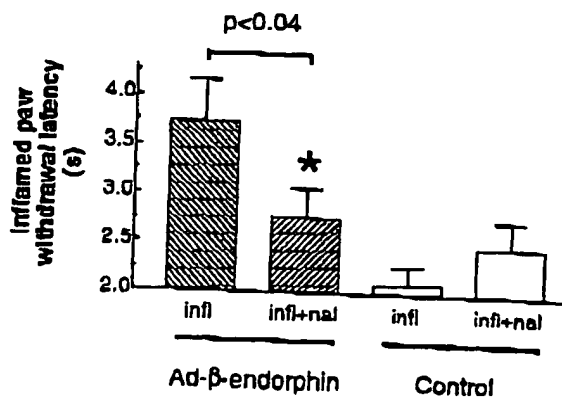


FIG. 4. Naloxone attenuation of Ad- $\beta$ -endorphin-mediated antihyperalgesia. 3–4 hours post inflammation, naloxone (2 mg/kg, intraperitoneal) was injected and latencies re-measured after an additional 40 min to allow drug dispersal ( $N = 18$ ).

digm for gene therapy approaches to central nervous system disorders. This paracrine paradigm has two key advantages for functional gene therapy. The first is the nonneuronal meningeal target, which provides for both a simple route of administration and allows manipulation of neurobehavioral function. The second key feature is spatial distribution of vector and secreted transgene product. Ad-lacZ spreads widely within the intrathecal space (Fig. 2D), demonstrating that the vector can distribute enough to modulate excitability over a multisegmental domain arising from primary afferent branching. This *in vivo* approach may need to be performed only once, with improvements in vector design for long-term, regulatable expression (Rivera *et al.*, 1996; A-Mohammadi and Hawkins, 1998; Hermens and Verhaagen, 1998; Saitoh *et al.*, 1998). This contrasts with intrathecal drug pumps, which require ongoing maintenance and pose an infection risk. Opioid genes may also be introduced into the peripheral nervous system, either directing secretion from primary afferent cells (Bras *et al.*, 1998), or directing local secretion from cells surrounding nociceptive nerve endings.  $\beta$ -Endorphin is effective against pain in models of knee inflammation and arthritis (Sluka and Westlund, 1993; Martinez *et al.*, 1996) and thus intraarticular injection of Ad- $\beta$ -endorphin, like intraarticular morphine (Stein *et al.*, 1991), may be a therapeutically useful synovial-paracrine approach to gene therapy for arthritis (Jorgensen and Gay, 1998) analogous to the present meningeal-paracrine approach.

A surprising finding from these experiments on viral delivery of  $\beta$ -endorphin concerns the specificity of action toward different types of pain. Morphine alters baseline thermal sensation at the same doses that affect inflammation-induced hyperalgesia (Ho *et al.*, 1997; Hargreaves *et al.*, 1988), i.e., in rats, morphine is analgesic. In contrast, the reduction in hyperalgesia observed with Ad- $\beta$ -endorphin was not accompanied by changes in baseline nociception, measured in the contralateral paw. Other opiate agonists such as bremazocine and GR89,696 also share this selectivity (Ho *et al.*, 1997), suggesting that the virally delivered peptide was acting at a similar opioid-sensitive site specifically related to hyperalgesia. Binding and functional data indicate that  $\beta$ -endorphin, bremazocine, and GR89,696 all recognize a non- $\mu$ , non- $\delta$ , non- $\kappa_1$  site in the spinal cord, termed  $\kappa_2$  (Ho *et al.*, 1997) or  $\epsilon$  (Nock *et al.*, 1993).  $\kappa_2/\epsilon$  sites exist in the spinal cords of a wide variety of species including humans (Caudle *et al.*, 1998), suggesting that the anti-hyperalgesic-specific effects of  $\beta$ -endorphin observed in this study may predict efficacy in chronic pain syndromes in humans.

The simplicity of this meningeal-paracrine gene therapy approach, rapidity of expression, ease of application, and apparent lack of side effects offer the possibility of a more general clinical utilization, especially in spinal cord injury or neurodegenerative diseases. Substitution of the  $\beta$ -endorphin sequence for growth factor genes (Barkats *et al.*, 1998; Tuszyński *et al.*, 1998) provides a means of supplying crucial factors to the spinal cord for increasing motor neuron survival in diseases such as spinal muscular atrophy or amyotrophic lateral sclerosis (Zurn *et al.*, 1998), or following spinal injury. Our results demonstrate *in vivo* delivery of the neuropeptide  $\beta$ -endorphin from a nonneuronal target cell, which then attenuates persistent pain behavior. This approach forms the basis for a novel ther-

apy for pain control, which can be extended to other neurological disorders.

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## REFERENCES

- A-MOHAMMADI, S., and HAWKINS, R.E. (1998). Efficient transgene regulation from a single tetracycline-controlled positive feedback regulatory system. *Gene Ther.* 5, 76-84.
- AMERICAN PAIN SOCIETY. (1995). Quality improvement guidelines for the treatment of acute pain and cancer pain. *JAMA* 274, 1874-1880.
- ANDERSON, W.F. (1998). Human gene therapy. *Nature (London)* 392, 25-30.
- BAJOCCHI, G., FELDMAN, S.H., CRYSTAL, R.G., and MASTRANGELI, A. (1995). Direct *in vivo* gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nature Genet.* 3, 229-234.
- BARKATS, M., BILANG-BLEUEL, A., BUC-CARON, M.H., CASTEL-BARTHE, M.N., CORTI, O., FINIELS, F., HORELLOU, P., REVAH, F., SABATE, O., and MALLET, J. (1998). Adenovirus in the brain: Recent advances of gene therapy for neurodegenerative diseases. *Prog. Neurobiol.* 55, 333-341.
- BECKER, T.C., NOEL, R.J., COATS, W.S., GOMER-POIX, A.M., ALAM, T., GERARD, R.D., and NEWGARD, C.B. (1994). Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* 43, 161-189.
- BERNABEI, R., GAMBASSI, O., LAPANE, K., LANDI, F., GATSONIS, C., DUNLOP, R., LIPSITZ, L., STEEL, K., and MOR, V. (1998). Management of pain in elderly patients with cancer. SAGE Study Group. Systematic Assessment of Geriatric Drug Use via Epidemiology. *JAMA* 279, 1877-1882.
- BEUTLER, A.S., BANCK, M.S., BACH, F.W., GAGE, F.H., PORRECA, F., BILSKY, E.J., and YAKSH, T.L. (1995). Retrovirus-mediated expression of an artificial beta-endorphin precursor in primary fibroblasts. *J. Neurochem.* 64, 475-481.
- BONICA, J.J. (1990). *The Management of Pain*. (Lea & Febiger, Philadelphia).
- BRAS, J.M.A., EPSTEIN, A.L., BOURGOIN, S., HAMON, M., CESSLIN, F., and POHL, M. (1998). Herpes simplex virus 1-mediated transfer of preproenkephalin A in rat dorsal root ganglia. *J. Neurochem.* 70, 1299-1303.
- CAUDLE, R.M., FINEGOLD, A.A., MANNES, A.J., TOBIAS, M.D., KENSHALO, D.R., and IADAROLA, M.J. (1998). Spinal kappa-1 and kappa-2 opioid binding sites in rats, guinea pigs, monkeys, and humans. *NeuroReport* 9, 2523.
- GUREJE, O., VON KORFF, M., SIMON, G.E., and GATER, R. (1998). Persistent pain and well-being: A World Health Organization study in primary care. *JAMA* 280, 147-151.
- HARGREAVES, K., DUBNER, R., BROWN, F., FLORES, C., and JORIS, J. (1988). A new and sensitive method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* 32, 77-88.
- HERMENS, W.T., and VERHAAGEN, J. (1998). Viral vectors, tools

- for gene transfer in the nervous system. *Prog. Neurobiol.* 55, 399-432.
- HO, J., MANNES, A.J., DUBNER, R., and CAUDLE, R.M. (1998). Putative kappa-2 opioid agonists are antihyperalgesic in a rat model of inflammation. *J. Pharmacol. Exp. Ther.* 281, 136-141.
- IADAROLA, M.J., LEE, S., and MANNES, A.J. (1997). Gene transfer approaches to pain control. *Molecular Neurobiology of Pain*. (IASP Press, Seattle, WA).
- INGHAM, J.M., and FOLEY, K.M. (1998). Pain and the barriers to its relief at the end of life: A lesson for improving end of life health care. *Hosp. J.* 13, 89-100.
- JACOBY, D.R., FRAEFEL, C., and BREAKFIELD, X.O. (1997). Hybrid vectors: A new generation of virus-based vectors designed to control the cellular fate of delivered genes. *Gene Ther.* 4, 1281-1283.
- JORGENSEN, C., and GAY, S. (1998). Gene therapy in osteoarthicular diseases: Where are we? *Immunol. Today* 19, 387-391.
- KISER, R.S., JACKSON, S., SMITH, R., REES, L.H., LOWRY, P.J., and BESSER, G.M. (1983). Endorphin-related peptides in rat cerebrospinal fluid. *Brain Res.* 288, 187-192.
- MANNES, A.J., CAUDLE, R.M., O'CONNELL, B.C., and IADAROLA, M.J. (1998). Adenoviral gene transfer to spinal-cord neurons: Intrathecal vs. intraparenchymal administration. *Brain Res.* 793, 1-6.
- MARTINEZ, J.H., MONDRAGON, C.E., and CESPEDAS, A. (1996). An evaluation of the antiinflammatory effects of intraarticular synthetic beta-endorphin in the canine model. *Anesth. Analg.* 82, 177-181.
- MAX, M.B., INTURRISI, C.E., KAIKO, R.F., GRABINSKI, P.Y., LI, C.H., and FOLEY, K.M. (1985). Epidural and intrathecal opiates: Cerebrospinal fluid and plasma profiles in patients with chronic cancer pain. *Clin. Pharmacol. Ther.* 38, 631-641.
- NAKAO, K., NAKAI, Y., OKI, S., MATSUBARA, S., KONISHI, T., NISHITANI, H., and IMURA, H. (1980). Immunoreactive beta-endorphin in human cerebrospinal fluid. *J. Clin. Endocrinol. Metab.* 50, 230-233.
- NOCK, B., GIORDANO, A.L., MOORE, B.W., and CICERO, T.J. (1993). Properties of the putative epsilon opioid receptor: Identification in rat, guinea pig, cow, pig and chicken brain. *J. Pharmacol. Exp. Ther.* 264, 349-359.
- OYAMA, T., JIN, T., YAMAYA, R., LING, N., and GUILLEMIN, R. (1980). Profound analgesic effects of beta-endorphin in man. *Lancet* 1, 122-124.
- RIVERA, V.M., CLACKSON, T., NATESAN, S., POLLOCK, R., AMARA, J.F., KEENAN, T., MAGARI, S.R., PHILLIPS, T., COURAGE, N.L., CERASOLI, P., JR., HOLT, D.A., and GILMAN, M. (1996). A humanized system for pharmacologic control of gene expression. *Nature Med.* 2, 1028-1032.
- SAGEV, J. (1998). Cellular transplantation for intractable pain. *Adv. Pharmacol.* 42, 579-582.
- SAITOH, Y., EGUCHI, Y., HAGIHARA, Y., ARITA, N., WATAHAKI, M., TSUJIMOTO, Y., and HAYAKAWA, T. (1998). Dose-dependent doxycycline-mediated adrenocorticotrophic hormone secretion from encapsulated Trp-on proopiomelanocortin Neuro2A cells in the subarachnoid space. *Hum. Gene Ther.* 9, 997-1002.
- SHIMOHAMA, S., ROSENBERG, M.B., PAGAN, A.M., WOLFF, J.A., SHORT, M.P., BREAKFIELD, X.O., FRIEDMANN, T., and GAGE, F.H. (1989). Grafting genetically modified cells into the rat brain: Characteristics of *E. coli* beta-galactosidase as a reporter gene. *Brain Res. Mol. Brain Res.* 5, 271-278.
- SLUKA, K.A., and WESTLUND, K.N. (1999). Behavioral and immunohistochemical changes in an experimental arthritis model in rats. *Pain* 55, 367-377.
- STEIN, C., COMISEL, K., HAIMERL, E., YASSOURIDIS, A., LEHRBERGER, K., HERZ, A., and PETER, K. (1991). Analgesic effect of intraarticular morphine after arthroscopic knee surgery. *N. Engl. J. Med.* 325, 1123-1126.
- TUSZYNSKI, M.H., WEIDNER, N., MCCORMACK, M., MILLER, I., POWELL, H., and CONNER, J. (1998). Grafts of genetically modified Schwann cells to the spinal cord: Survival, axon growth, and myelination. *Cell Transplant.* 7, 187-196.
- WALL, P.D., and MELZACK, R. (1994). *Textbook of Pain*. (Churchill Livingstone, New York).
- WU, H.H., WILCOX, G.L., and McLOON, S.C. (1994). Implantation of A $\delta$ -20 or genetically modified A $\delta$ -20/hENK cells in mouse spinal cord induced antinociception and opioid tolerance. *J. Neurosci.* 14, 4806-4814.
- YAKSH, T.L., and HENRY, J.L. (1978). Antinociceptive effects of intrathecally administered human beta-endorphin in the rat and cat. *Can. J. Physiol. Pharmacol.* 56, 754-759.
- ZURN, A.D., TSENG, J.L., DEGLON, N., JOSEPH, J.M., and AB-BISCHER, P. (1998). A gene therapy approach for the treatment of amyotrophic lateral sclerosis and Parkinson's disease. *Adv. Pharmacol.* 42, 929-931.

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# Therapeutic Efficacy in Experimental Polyarthrititis of Viral-Driven Enkephalin Overproduction in Sensory Neurons

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Rheumatoid arthritis is characterized by erosive inflammation of the joints, new bone proliferation, and ankylosis, leading to severely reduced locomotion and intense chronic pain. In a model of this disease, adjuvant-induced polyarthrititis in the rat, neurons involved in pain transmission and control undergo plastic changes, especially at the spinal level. These changes affect notably neurons that contain opioids, such as enkephalins deriving from preproenkephalin A (PA) precursor protein. Using recombinant herpes simplex virus containing rat PA cDNA, we enhanced enkephalin synthesis in sensory neurons

of polyarthritic rats. This treatment markedly improved locomotion and reduced hyperalgesia. Furthermore, the progression of bone destruction slowed down, which is the most difficult target to reach in the treatment of patients suffering from arthritis. These data demonstrate the therapeutic efficacy of enkephalin overproduction in a model of systemic inflammatory and painful chronic disorder.

**Key words:** proenkephalin A overproduction; dorsal root sensory ganglia neurons; polyarthritic rats; reduced hyperalgesia; improved polyarthrititis-related disability; limited joint destruction

Rheumatoid arthritis is a systemic autoimmune disease primarily manifested by chronic erosive inflammation of the joints associated with intense pain (Pearson, 1956; Colpaert, 1987; Harris, 1990). Its etiology is still unknown, but significant insights into its physiopathology have been obtained from experimental animal models. Although none of these models has all of the characteristics of the human disease, adjuvant-induced polyarthrititis in the rat shares numerous behavioral and biochemical characteristics with rheumatoid arthritis (Pearson, 1956; Calvino et al., 1987; Colpaert, 1987).

Proenkephalin A (PA)-derived peptides are involved in the control of pain, and an antihyperalgesic action of overexpressed PA was reported recently in experimentally induced acute inflammatory pain in the mouse (Wilson et al., 1999). Numerous data indicate that opioids, in addition to acting at central sites, modulate pain and inflammatory processes by acting at peripheral sites (Stein and Yassouridis, 1997; Houghton et al., 1998). At the periphery, opioid peptides are thought to originate mainly from inflammatory cells (Schäfer et al., 1994); however, although PA is expressed in a relatively slight proportion of cell bodies of sensory neurons (Pohl et al., 1994), which are located in dorsal root ganglia (DRG), enkephalin-containing axons and terminals present within glabrous skin (Carlton and Coggeshall, 1997) and soft tissue of joints (El Hassan et al., 1998) might represent an

additional source of peripheral opioids. Interestingly, in polyarthritic rats, PA expression drops in lumbar DRG that contain cell bodies of sensory nerves from hindlimbs (especially affected by the disease) (Pohl et al., 1997), and the levels of the main PA-derived peptide, met-enkephalin (ME), are reduced in the soft tissue of ankle joints (El Hassan et al., 1998).

Altogether, these findings led us to assess whether PA overexpression in sensory neurons of the hindlimbs could have a beneficial effect in chronically suffering polyarthritic rats. We used herpes simplex virus type 1 (HSV-1)-derived vectors, particularly suitable for transgene transfer into sensory neurons (Geller and Breakefield 1988; Davar et al., 1994; Smith et al., 1995; Goins et al., 1999). We demonstrated recently these vectors to be highly efficient to drive PA gene expression in DRG neurons of healthy rats (Antunes-Bras et al., 1998, 2001). Here, we generated recombinant, rat PA cDNA containing thymidine kinase-defective HSV-1 vectors (HSVLatEnk) to prevent possible viral replication and spread.

## MATERIALS AND METHODS

**HSV-derived vector construction.** The pLatEnk plasmid was constructed by subcloning under the Lat-long terminal repeat (LTR) promoter (Lokensgard et al., 1994) the rat PA coding region (derived from pYSEA1; Yoshikawa et al., 1984) into the *HindIII*-*Eco47III*-linearized pLat-LTR-LacZ vector (Antunes-Bras et al., 1998). Thymidine kinase gene (*UL<sub>23</sub>*) in KOS HSV-1 DNA, bearing the Lat-LTR-LacZ transcriptional units in *gC* locus (*Tk*<sup>+</sup>HSVLat $\beta$ -gal; Antunes-Bras et al., 1998), was disrupted by homologous recombination with p23d plasmid containing deleted *UL<sub>23</sub>* gene (520 bp *NsiI* to *SacI* fragment). *Tk*<sup>−</sup>HSVLat $\beta$ -gal was generated by cotransfecting 5  $\mu$ g of linearized p23d DNA, using the calcium phosphate precipitation method, with 5  $\mu$ g of HSVLat $\beta$ -gal into Vero cells grown in 1 $\times$  DMEM (Life Technologies, Gaithersburg, MD) containing 10 U/ml of penicillin and streptomycin, 7.5 mM sodium bicarbonate, 2 mM glutamine, and 10% fetal calf serum. Cell cultures were treated 4 hr later with 15% glycerol (v/v) and incubated for 4 d at 37°C in M199 medium (Life Technologies) until the cytopathic effect had spread

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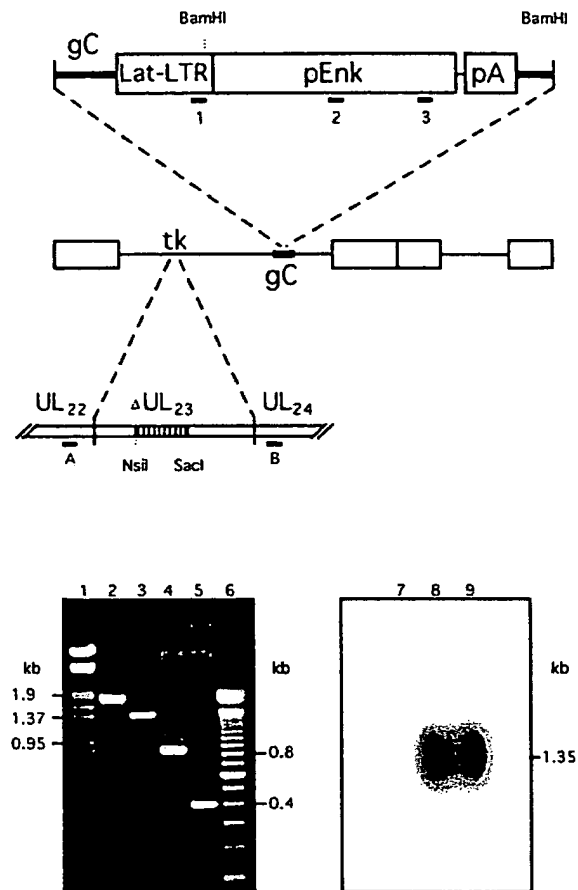
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throughout the cellular monolayer. Cellular debris were spun down, and new confluent Vero cells were infected with the resulting supernatant and incubated at 37°C in the presence of 10  $\mu$ M acyclovir. Lysis plaques resistant to acyclovir were picked and separated in two aliquots. One aliquot was saved, and DNA was extracted from the second one and screened for the presence of deleted UL<sub>23</sub> gene using PCR analysis. Thirty PCR cycles (96°C 1 min; 60°C 0.5 min; and 72°C 2 min) were made with 40 pmol of primers (primer A, 5'-GCGCTCCTCGTACCAGC-GAAG3'; primer B, 5'-CCAGCGTCTTGTCAT TGGCG3') (Fig. 1) in a mixture containing 10 mM each dNTP, 25 mM MgSO<sub>4</sub>, and 0.5 U of *Taq* DNA polymerase (Eurobio, Les Ulis, France), in 1× reaction buffer containing 2.5% formamide. Single plaque-isolated Tk<sup>−</sup>HSVLat $\beta$ -gal (HSVLat $\beta$ -gal) were then amplified on Vero cells. HSVLatEnk was generated by pLatEnk DNA homologous recombination with HSVLat $\beta$ -gal DNA in Vero cells as indicated above. Recombinant HSVLatEnk were isolated by PCR analysis and purified by successive limiting dilutions. The presence of both the disrupted TK gene and the Lat-LTR-pEnk transcriptional unit (primer 1, 5'-CTGACTGTGTTTCTGTAT-TTG3'; primer 2, 5'-TAGCCAAGAAGTATGGAGGG3'; primer 3, 5'-TGATAGTCCATC CACCACTCG3') was confirmed by PCR analysis (using the same experimental protocol as above) on purified HSV-LatEnk DNA obtained from plaque isolates (Fig. 1). Finally, HSV-LatEnk DNA was further analyzed by Southern blot hybridization with [<sup>32</sup>P]-labeled cDNA probe corresponding to the entire coding region of rat PA mRNA (Fig. 1). Briefly, 10  $\mu$ g of purified HSVLatEnk DNA, 10  $\mu$ g of HSVLat $\beta$ -gal, and 0.1  $\mu$ g of pLatEnk plasmid DNA were *Bam*HI digested and separated on 0.8% agarose gel. After DNA denaturation and gel neutralization, DNA was electrically transferred onto Hybond N nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). After prehybridization (3 hr, 42°C) in 50 mM sodium phosphate buffer, pH 6.5, containing 50% formamide, 0.5% SDS, 5× SSC, 5× Denhardt's solution, and 0.2 mg/ml denatured herring sperm DNA, the blot was hybridized overnight at 42°C in the same solution with  $\sim 1.5 \times 10^6$  cpm/ml [<sup>32</sup>P]-labeled cDNA probe. Membrane was gradually washed with 2× SSC containing 0.5% SDS at room temperature and then at 45°C, followed by 0.1% SSC at 65°C. The blot was finally exposed to x-ray film (Hyperfilm MP; Amersham Pharmacia Biotech) for 3–5 hr at room temperature.

HSVLatEnk and HSVLat $\beta$ -gal were concentrated by ultracentrifugation (50,000 × g; 4°C; 1 hr). Viruses were resuspended in 10% sucrose, and their titers were determined using standard plaque assay on Vero cells. The HSVLatEnk and HSVLat $\beta$ -gal titers were of  $5 \times 10^8$  pfu/ml and  $2 \times 10^9$  pfu/ml, respectively. Functional efficacy of HSVLatEnk was characterized *in vitro* on either Vero cells or human neuroblastoma cells (SK-N-MC) by searching for the presence of PA encoding mRNA and ME-like material (MELM) in both infected (with either HSVLatEnk or HSVLat $\beta$ -gal) and uninfected cells. Briefly, 48 hr after infection of cells at a multiplicity of infection of 1, total RNA was extracted using the acid-guanidinium method (Chomczynski and Sacchi, 1987) and treated with DNase. RNA was then recovered by phenol–chloroform extraction and ethanol precipitation. Proenkephalin A mRNA was detected by reverse transcription (RT)-PCR on 0.5  $\mu$ g of total RNA according to the Access RT-PCR system instructions (Promega, Madison, WI) using 40 pmol of each PA-specific primer (5'-TAGCCAAGAAGTATGGAGGG3'; and 5'-GACTATCAGGTAGGTGGTGGAGC3'). Synthesis of ME-like material was revealed using immunohistochemistry with a monoclonal anti-ME antibody (1:1000; Valbiotech, Paris, France). Forty-eight hours after infection, cells on poly-D-lysine-coated coverslips were fixed with 4% paraformaldehyde in PBS at room temperature. After washing, cells were preincubated in PBS containing 0.1% Triton X-100 and 6% normal donkey serum (Jackson ImmunoResearch, West Grove, PA) and then processed as described in the protocol for immunohistochemistry (see below).

**Animals and treatments.** All experiments were performed in accordance with institutional guidelines that are in compliance with national and international law and policies for use of animals in neuroscience research (Council directive number 87/48, Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale). All animals were maintained under the same conditions (22  $\pm$  1°C; 60  $\pm$  10% relative humidity; 12 hr light/dark cycle; food and water available *ad libitum*). Polyarthrititis was induced by an intradermal injection at the base of the tail of 0.05 ml of Freund's adjuvant (Gouret et al., 1976) in 6-week-old male Sprague Dawley rats. In addition to healthy animals, three homogeneous groups of polyarthritic rats were constituted and deeply anesthetized with chloral hydrate (400 mg/kg, i.p.) for virus or



**Figure 1.** Schematic representation of recombinant HSVLatEnk vector and its molecular characterization. Thymidine kinase gene (UL<sub>23</sub>) in KOS-derived Tk<sup>+</sup>HSVLat $\beta$ -gal DNA [bearing  $\beta$ -galactosidase reporter gene downstream of the Lat-LTR promoter inserted into the gC locus (Antunes-Bras et al., 1998)] was disrupted by homologous recombination with p23d plasmid DNA containing *Nsi*I to *Sac*I deleted HSV TK gene to generate TK-negative HSVLat $\beta$ -gal vector (HSVLat $\beta$ -gal). HSVLatEnk recombinant was then created as described previously (Antunes-Bras et al., 1998), by inserting Lat-LTR-pEnk transcriptional unit into the gC gene of HSVLat $\beta$ -gal by homologous recombination. Purified HSV-LatEnk DNA was analyzed by PCR, and subsequent PCR products were separated on ethidium bromide-stained 1.2% agarose gel for the presence of both the TK-deleted gene and the Enk transgene. Positions of respective primers used are indicated on the diagram. Lanes 1 and 6 show the Lambda DNA/*Hind*III-*Eco*RI and 100 bp molecular weight standards (given in kilobases), respectively. Lane 2, Approximately 1.8 kb PCR product generated by amplification, using primers A/B, of wild-type HSV DNA; lane 3,  $\sim 1.3$  kb PCR product generated using the same primers A/B on HSVLatEnk DNA. Lanes 4 and 5 show the PCR amplification products obtained with the set of primers 1/3 and 2/3 on HSVLatEnk DNA, respectively. HSVLatEnk DNA was further analyzed by Southern hybridization. Ten micrograms of HSVLat $\beta$ -gal DNA (lane 7), 10  $\mu$ g of HSVLatEnk DNA (lane 8), and 0.1  $\mu$ g of pLatEnk DNA (lane 9) were digested with *Bam*HI, applied on 0.8% agarose gel, electrotransferred on nylon membrane, and hybridized with a [<sup>32</sup>P]-labeled cDNA probe corresponding to the rat pEnk coding region. Whereas no hybridization signal was apparent on HSVLat $\beta$ -gal (lane 7), positively labeled DNA fragments of  $\sim 1.35$  kb were generated on HSVLatEnk DNA (lane 8) and pLatEnk DNA (lane 9). This size corresponds to the expected size of DNA fragment resulting from the *Bam*HI digestion of both HSVLatEnk and pLatEnk DNA.

vehicle administration. The first and second group of polyarthritic rats were infected bilaterally on slightly scarified hind footpads with  $\sim 5 \times 10^6$  pfu of either HSVLatEnk or HSVLat $\beta$ -gal, respectively. Treatment of sham-infected polyarthritic rats in the third group consisted of vehicle (10  $\mu$ l of 10% sucrose in 0.9% NaCl) application onto scarified footpads.

Previous studies showed that the resulting increase in the concentrations of MELM in rat lumbar DRG is maximum 3 weeks after infection with HSVLatEnk (Antunes-Bras et al., 1998). Because most of the polyarthritic-associated symptoms peak 3–5 weeks after induction of the disease (Calvino et al., 1987), rats were infected 2 weeks after polyarthritic induction, and subsequent experiments were generally performed 3 weeks later. In particular, animals used for radioimmunoassay, immunohistochemical, or *in situ* hybridization procedures were killed by decapitation 3 weeks after infection, i.e., 5 weeks after polyarthritic induction. The lumbar enlargement of the spinal cord and adjacent DRG (L1–L6) were dissected at 0–4°C. The spinal cord was divided into its ventral and dorsal parts (except when used for *in situ* hybridization experiments), and left and right ganglia were pooled. Tissue pieces for RNA analyses were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$ . Radioimmunological determinations of tissue levels of ME, calcitonin gene-related peptide (CGRP), and substance P were performed as described previously (Cesselin et al., 1980; Pohl et al., 1990).

**Hindpaw diameter measurements and radiological analyses.** Animals were anesthetized with chloral hydrate (300 mg/kg, i.p.) 2 weeks (i.e., just before infection with either HSVLat $\beta$ -gal or HSVLatEnk) and 5 weeks after polyarthritic induction. The diameter of their hindpaws was measured using a digital micrometer (Mitutoyo). The presence and severity of lesions were evaluated on radiographs (made 2, 3, and 5 weeks after polyarthritic induction) by an expert rheumatologist, who was unaware of the treatments. A four-degree rating scale was used for the bilateral evaluation of ankle and metatarsus toe joints: 0, no obvious lesions; 1, doubtful or mild lesions; 2, medium lesions with joint space narrowing or disappearance; and 3, severe lesions with joint destruction and mild periostitis. To take into account as precisely as possible the development of periostitis, which is an important variable in joint lesions (and can be widely developed despite relatively medium joint destruction), each score value was raised by one if extensive periostitis was present. Accordingly, the maximum rate was 16. Because no differences in hindpaw diameters or severity scores of lesions were observed between sham-infected and HSVLat $\beta$ -gal-infected polyarthritic rats, animals in these two groups are referred to as control polyarthritic rats.

**Behavioral studies.** Pain-related behavior was assessed by measuring the latency of foot withdrawal elicited by noxious radiant heat (Ugo Basile Plantar test, intensity 7; Ugo Basile, Comerio, Italy) applied to hindpaw plantar surface (Galbraith et al., 1993). Three measures at 1 hr intervals were performed bilaterally, and the mean was considered as one value for each animal.

Spontaneous locomotor activity was measured in an open field, the floor of which was divided into five compartments with black lines. The open field was in a red-lighted room in which animals were introduced 12 hr before the beginning of the experiments. Each animal was placed into the open field, and 1 min later its locomotor activity was video monitored and tape recorded. The number of line crosses and rearings were counted for a 7 min period by two independent observers in a distant room.

Performances of the different groups of polyarthritic rats were first assessed 3 weeks after infection and compared with those of healthy controls. The next day, animals were anesthetized with chloral hydrate (300 mg/kg, i.p.). The skin was incised at the level of the scapula, an Alzet osmotic minipump (delivery rate of 1  $\mu$ l/hr; 2001 model; Alza Scientific Products, Palo Alto, CA) was implanted subcutaneously, and the incision was then sutured. Following the instructions of the manufacturer, osmotic minipumps were filled with either naloxone or naloxone methiodide to administer each of these drugs at the dose of 3 mg  $\cdot$  kg $^{-1}$   $\cdot$  d $^{-1}$ . "Sham"-treated animals were implanted with saline-delivering minipumps. Behavioral studies were performed on the fourth day after minipump implantation. Observers were blinded to the group of polyarthritic rats (either controls or HSVLatEnk-infected rats) and to the drug delivered. Similar conditions were used to assess the performances of animals 5 and 8 weeks after infection. Behavioral experiments did not reveal any differences between sham-infected and HSVLat $\beta$ -gal-infected polyarthritic rats.

**In situ hybridization.** Deeply anesthetized animals (chloral hydrate; 400 mg/kg, i.p.), were perfused transcardially with 100 ml of saline (0.9% NaCl) supplemented with 0.1% sodium nitrite, followed by 600 ml of 4% paraformaldehyde in PBS, at room temperature. Spinal cord and L1–L6

dorsal root ganglia were removed, post-fixed for 2 hr in the same solution at  $4^\circ\text{C}$ , cryoprotected in 10% sucrose–PBS, frozen, and stored at  $-80^\circ\text{C}$  until used. Ten micrometer cryostat sections were fixed for 1 hr with 4% paraformaldehyde in PBS at  $4^\circ\text{C}$ , rinsed in PBS, and dehydrated through a graded series of ethanol concentrations (30–100%). Slides with tissue sections were incubated in the presence of a cRNA probe of latency-associated transcripts (LATs) labeled with digoxigenin-11-UTP according to the instructions of the manufacturer (Promega, Madison, WI). This probe (188–621 bases PCR amplified segment of HSV-1 LAT sequence; Wagner et al., 1988) allows the detection of all viral particles, independent of their ability to express the PA transgene. Hybridization was performed overnight at  $65^\circ\text{C}$  in  $1\times$  SSC, 50% formamide, 10% dextran sulfate, 1 mg/ml rRNA, and  $1\times$  Denhardt's solution. On the following day, sections were washed twice with  $1\times$  SSC, 50% formamide, and 0.1% Tween 20, at  $65^\circ\text{C}$ , and twice with 100 mM maleic acid, 150 mM NaCl, and 1% Tween 20, at room temperature. The digoxigenin-labeled hybrids were detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody following the instructions of the manufacturer (Roche Products, Hertfordshire, UK). PA mRNA was detected by hybridizing tissue sections with  $\sim 3.3 \times 10^6$  cpm/ $\mu$ l antisense [ $^{32}\text{S}$ ]cRNA (corresponding to the entire coding region of the rat preproenkephalin A cDNA) as described in detail previously (Pohl et al., 1994).

**Quantitative RT-PCR.** Total RNA, extracted as described above, was quantified using as reference a scale of total RNA prepared on cesium chloride gradient and estimated from the optical density at 260 nm. RT-PCR was performed, as described previously (Antunes-Bras et al., 1998), with 0.5  $\mu$ g of each RNA sample in the presence of various amounts (0.5–16 fg) of internal synthetic standard prepared according to the PCR MIMIC construction kit (Clontech, Cambridge, UK). This 241 bp standard fragment, flanked with PA sequences (21 bp), was amplified with the same set of rat PA-specific primers as the cDNA. Reverse-transcribed RNA was amplified with 30 cycles (96, 58, and  $72^\circ\text{C}$ ; 1 min each) according to the Access RT-PCR system instructions (Promega) using 40 pmol of primers in a mixture containing 10 mM of each dNTP, 25 mM MgSO $_4$ , 2.5 U of avian myeloblastosis virus reverse transcriptase, 2.5 U of thermus flavus DNA polymerase, and 6.5 U of RNase inhibitor (RNasin) in  $1\times$  reaction buffer. The RT-PCR products were electrophoresed on 1.2% ethidium bromide-stained agarose gel and quantified with the gel analyzer GDS 5000 (Ultra-Violet Products Ltd., Cambridge, UK).

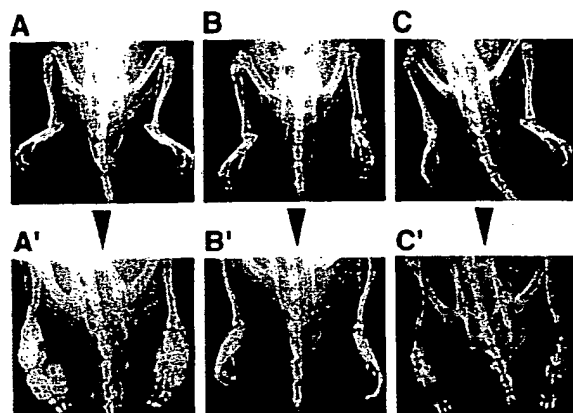
**Immunohistochemistry.** Animals were anesthetized and perfused as described in the protocol for *in situ* hybridization. Segments of sciatic nerve, L4–L5 dorsal roots, and L4–L5 DRG were dissected out and cryoprotected in 10% sucrose (24 hr,  $4^\circ\text{C}$ ). Fifteen micrometer cryostat sections were preincubated (30 min, room temperature) in PBS containing 0.3% Triton X-100 and 3% normal donkey serum (Jackson ImmunoResearch) and then incubated overnight at  $4^\circ\text{C}$  in the same buffer supplemented with a monoclonal anti-ME antibody (1:1000; Valbiotech). After washing in PBS, sections were incubated for 1 hr with rhodamine (Cy3)-conjugated anti-mouse Ig (1:800; Interchim, Montluçon, France), rinsed in PBS, mounted in fluoromount-G (CliniSciences, Montrouge, France), and examined using a Leica (Nussloch, Germany) confocal microscope. Both labeled and unlabeled neurons were counted in every fourth sections of a total of  $\sim 30$  sections for each DRG (L4–L5, unilaterally), in three animals per group. A total of 712–852 neurons were analyzed in each group.

**Statistical analyses.** All data, including those from behavioral experiments, were subjected to the unpaired Student's *t* test. The performances of rats after versus before treatment with opioid receptor antagonists were compared using the paired Student's *t* test. The Kolmogorov–Smirnov test was used for statistical analysis of the data derived from clinical evaluation of the joint lesions. When  $p > 0.05$ , the corresponding difference was considered to be not significant.

## RESULTS

### Morphological aspects and osseous lesions of hindpaws in HSVLatEnk-infected polyarthritic rats

In agreement with previous reports (Pearson, 1956; De Castro Costa et al., 1981), soft tissue swelling and increased joint diameter of hindpaws were apparent in nearly 100% of animals 2 weeks after polyarthritic induction (bilateral hindpaw joint diameter in control polyarthritic rats,  $13.3 \pm 1.2$  mm, mean  $\pm$  SEM,  $n = 18$ ; versus  $9.0 \pm 0.1$  mm in healthy rats,  $n = 8$ ;  $p < 0.01$ ). In

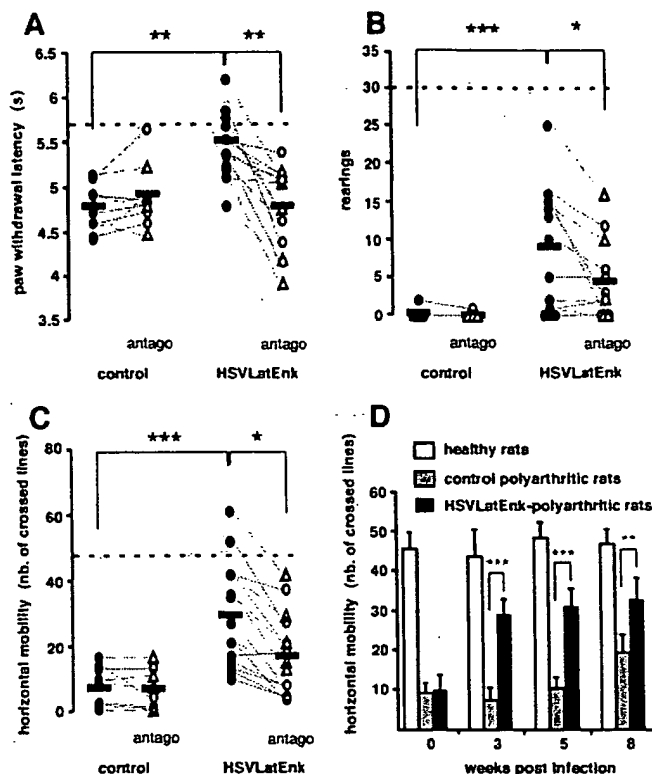


**Figure 2.** Presence and severity of hindpaw joint lesions in control (representing both sham-infected or HSVLat $\beta$ -gal-infected polyarthritic rats) (*A'*) and HSVLatEnk-infected (*B'*, *C'*) polyarthritic rats. Individual evolution of polyarthritis-associated joint destruction was assessed by comparing radiograms made 2 weeks after polyarthritis induction (*A*, *B*, *C*), i.e., just before infection with either HSVLat $\beta$ -gal or HSVLatEnk, and then 3 weeks later (*A'*, *B'*, *C'*). Osseous lesions of tarsus and metatarsus toe were evaluated bilaterally in both groups of rats ( $n = 8$ – $10$ ) using a four-degree rating scale. Radiographs were examined by an expert observer, without knowledge of the treatments. The Kolmogorov–Smirnov test was used for statistical analysis of the data. Six of 10 HSVLatEnk-infected rats presented mild lesions (*B'*), and the remaining four animals had more extensive lesions (*C'*), which were, however, less important than those of control polyarthritic rats (*A'*).

control polyarthritic rats, these signs of inflammation worsened for the following weeks, with larger hindpaw swelling and joint diameter ( $17.4 \pm 1.0$  mm;  $n = 8$ ;  $p < 0.02$ ) at the end of the fifth week. In contrast, in paired HSVLatEnk-infected polyarthritic rats, the hindpaw joint diameter did not enlarge from the second to the fifth week ( $13.1 \pm 1.0$  mm;  $n = 8$ ) after polyarthritis induction. Just before infection by either HSVLat $\beta$ -gal or HSVLatEnk, i.e., 2 weeks after polyarthritis induction, radiogram analysis yielded scores of hindpaw joint lesions ranging from 6 to 10 (Fig. 2*A–C*). Three weeks later, marked lesions, including extensive erosion of bone extremities, narrowing or disappearance of joint spaces, calcification, and new bone proliferation, were observed in control animals ( $13 \leq$  lesion scores  $\leq 16$ ) (Fig. 2*A'*). Radiogram analysis of HSVLatEnk-infected rats (Fig. 2*B', C'*) revealed that the mean scores of their hindpaw joint lesions were significantly lower ( $p < 0.001$ ;  $n = 10$ ) than those in control polyarthritic rats (Fig. 2*A'*). Despite inter-individual variability in polyarthritis development; 6 of the 10 HSVLatEnk-infected rats examined presented only medium lesions of the hindpaw joints (lesion scores  $\leq 9$ ) (Fig. 2*B'*). The other four HSVLatEnk-infected animals had extensive lesions ( $9 <$  lesion scores  $\leq 11$ ) (Fig. 2*C'*), which never reached the severity of joint destruction regularly observed in control polyarthritic rats. Treatment with naloxone methiodide ( $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) for 3 d did not affect the radiological lesion scores in HSVLatEnk-infected polyarthritic rats (data not shown).

#### Effects of HSVLatEnk infection on pain-related behavior in polyarthritic rats

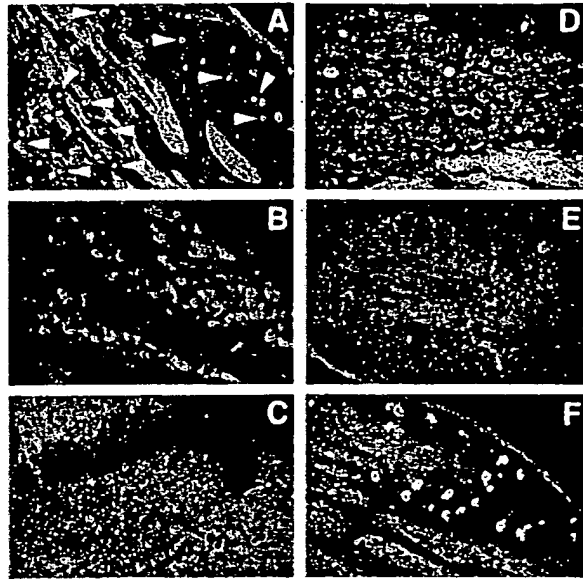
Compared with healthy rats (latency of foot withdrawal to radiant heat,  $5.7 \pm 0.3$  sec; mean  $\pm$  SEM;  $n = 7$ ), polyarthritic control rats had slightly reduced latency 5 weeks after polyarthritis induction ( $4.8 \pm 0.2$  sec;  $p < 0.02$ ;  $n = 15$ ). At the same time (i.e., 3 weeks after injection), the paw withdrawal latencies of HSVLatEnk-



**Figure 3.** HSVLatEnk-infected polyarthritic rats exhibited reduced thermal hyperalgesia and improved spontaneous locomotor activity. Response (paw withdrawal) latencies (*A*) of controls ( $n = 9$ ) and HSVLatEnk-infected ( $n = 15$ ) polyarthritic rats to radiant heating (intensity 7; Ugo Basile) were measured 3 weeks after infection. Rearings (*B*) and horizontal locomotor activity (*C*) of control (sham- or HSVLat $\beta$ -gal-infected) ( $n = 12$ ) and HSVLatEnk-infected ( $n = 15$ ) polyarthritic rats in a red-lighted open field were video monitored and assessed every minute during a 7 min period. Animals were then implanted subcutaneously for 3 d with an Alzet osmotic minipump delivering  $3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$  of either naloxone (O) or naloxone methiodide ( $\Delta$ ) (*antago*), and thermal hyperalgesia and locomotor activity were assessed. Performances of normal healthy rats are indicated by the horizontal dashed line and gray band in the three behavioral tests (mean  $\pm$  SEM;  $n = 6$ – $7$ ). \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  for control versus HSVLatEnk-infected polyarthritic rats, two-tailed unpaired *t* test; and for untreated versus naloxone–naloxone methiodide-treated animals, two-tailed paired *t* test. Long-term improvement of functional ability (*D*) in HSVLatEnk-infected polyarthritic rats was evaluated by comparing the locomotor activity in normal healthy rats ( $n = 5$ ) and in both control ( $n = 8$ ) and HSVLatEnk-infected ( $n = 10$ ) polyarthritic rats 3, 5, and 8 weeks after infection. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$  for control versus HSVLatEnk-infected rats; two-tailed unpaired *t* test.

infected animals were significantly higher than those of control polyarthritic rats ( $5.5 \pm 0.2$  sec;  $p < 0.001$ ;  $n = 15$ ) (Fig. 2*A*). Measurement of spontaneous locomotor activity of healthy rats indicated that they performed  $48 \pm 7$  (mean  $\pm$  SEM;  $n = 6$ ) crosses of the lines and  $30 \pm 4$  (mean  $\pm$  SEM;  $n = 6$ ) rearings for the 7 min recording period. Five weeks after induction of the disease, both the horizontal locomotor activity ( $7 \pm 2$  crosses; mean  $\pm$  SEM;  $n = 11$ ) and rearings ( $0.2 \pm 0.1$ ; mean  $\pm$  SEM;  $n = 11$ ) were dramatically reduced in control polyarthritic rats (Fig. 3*B, C*). Compared with the latter animals, paired HSVLatEnk-infected rats showed a remarkable improvement in both rearings (by eightfold;  $p < 0.001$ ;  $n = 12$ ) (Fig. 3*B*) and horizontal displacements (by 4.2-fold;  $p < 0.001$ ;  $n = 15$ ) (Fig. 3*C*) 3 weeks





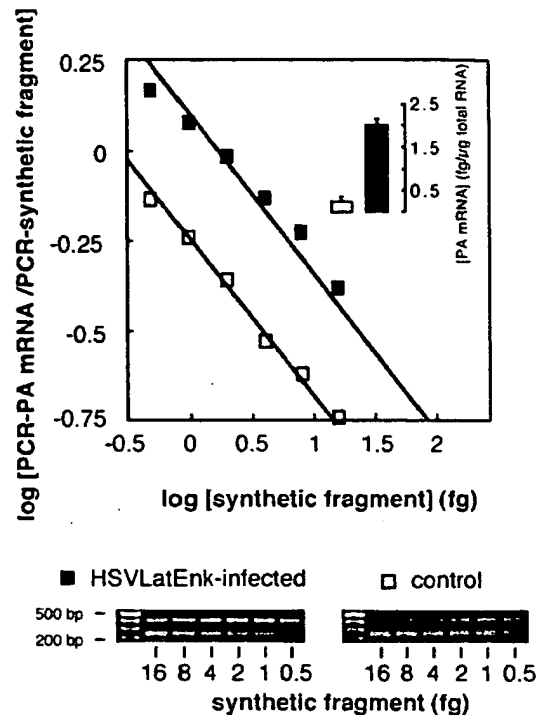
**Figure 4.** LATs and PA mRNA expression were detected by *in situ* hybridization histochemistry. Incubation of 10  $\mu$ m sections of L4–L5 DRG with digoxigenin-labeled LAT cRNA revealed numerous LAT-expressing neurons (arrowheads) in HSVLatEnk-infected polyarthritic rats (A). In contrast, no LAT-expressing nerve somas could be detected in adjacent L1–L3 DRG (B) or in the spinal cord of the lumbar region (C), suggesting no spread of the vectors to these territories. Every fourth section of L4–L5 DRG from normal healthy rats (D), control (E), or HSVLatEnk-infected polyarthritic rats (F) was incubated with [ $^{35}$ S]PA cRNA, and then both unlabeled and labeled neurons were counted (in a total of 30 sections in each group). Approximately 2% of neurons in L4–L5 DRG of healthy rats were labeled with [ $^{35}$ S]PA cRNA (D). In contrast, no PA mRNA-expressing neurons were detected in L4–L5 DRG of control (vehicle- or HSVLat $\beta$ -gal-infected) polyarthritic rats (E). Infection with HSVLatEnk led to the expression of PA mRNA in ~12% (110 of a total of 955 neuronal somas) of neurons in L4–L5 DRG (F). The photomicrographs are representative of three to four animals per group, examined 5 weeks after polyarthritis induction (i.e., 3 weeks after vehicle, HSVLat $\beta$ -gal, or HSVLatEnk infection). Scale bar: A, B, D–F, 250  $\mu$ m; C, 390  $\mu$ m.

after infection. This amelioration persisted during the whole observation period, i.e., for at least 8 weeks after HSVLatEnk infection (Fig. 3D).

Behaviors were not significantly changed in both control and HSVLatEnk-infected polyarthritic rats implanted with saline-containing minipumps. Similarly, naloxone or naloxone methiodide, administered at the dose of 3 mg  $\cdot$  kg $^{-1}$   $\cdot$  d $^{-1}$  for 3 d, were without any significant effect in control polyarthritic rats. In contrast, both compounds reversed with a similar efficacy both the antihyperalgesic response ( $p < 0.001$ ;  $n = 15$ ) (Fig. 3A) and the improved locomotor activity ( $p < 0.05$ ;  $n = 12$ –15) (Fig. 3B,C) in HSVLatEnk-infected animals.

#### L calization and quantification of HSVLatEnk-mediated ME overexpression.

*In situ* hybridization histochemistry with a digoxigenin-labeled complementary probe of HSV LATs showed that, after the peripheral infection of hindpaws in polyarthritic rats, viral vectors were restricted to L4–L5 DRG (Fig. 4A). This indicated rapid entry in latency and no spread of vectors to adjacent DRG or the spinal cord (Fig. 4B,C, respectively). *In situ* hybridization with [ $^{35}$ S]-labeled rat PA complementary probe showed that only

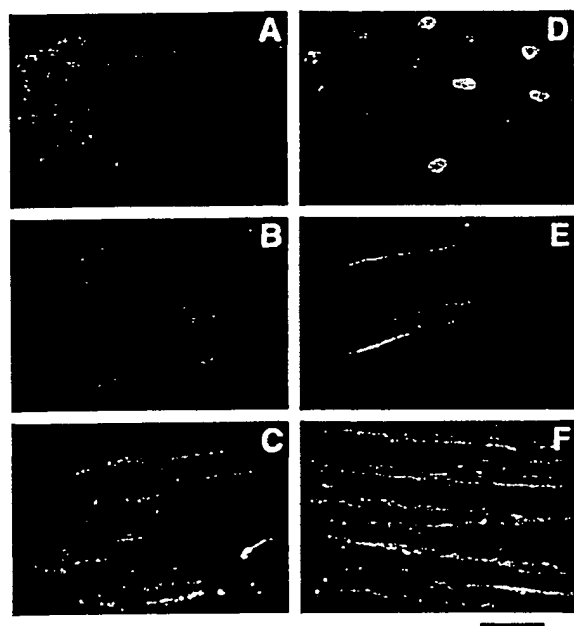


**Figure 5.** Quantitative RT-PCR measurement of PA mRNA levels in L4–L5 DRG of control (□) or HSVLatEnk-infected (■) polyarthritic rats. Total RNA was extracted from L4–L5 DRG in both groups of animals ( $n = 5$  for each group) 5 weeks after polyarthritis induction. Five hundred nanograms of total RNA were reverse-transcribed in the presence of six different dilutions of synthetic fragment and amplified for 30 cycles. Measurement of optical density of PCR amplification products of PA mRNA (402 bp) or of the synthetic fragment (241 bp) allowed the drawing of the plot, as described previously (Antunes-Bras et al., 1998). Representative gel analyses of PCR products are shown.

~2–3% of neurons express PA mRNA in L4–L5 DRG of healthy rats (Fig. 4D). In control polyarthritic rats, no or only scarce PA mRNA-expressing cells were detected in lumbar L4–L5 DRG (Fig. 4E). In contrast, peripheral inoculation of polyarthritic rats with HSVLatEnk led to a significant increase in the number of PA mRNA-expressing neurons in L4–L5 DRG (Fig. 4F), reaching ~12% of the total neuron population. Quantitative RT-PCR allowed the demonstration that PA mRNA levels in L4–L5 DRG of HSVLatEnk-infected rats were approximately eightfold higher ( $p < 0.001$ ;  $n = 5$ ) than in control polyarthritic animals (Fig. 5). In addition, MELM concentrations in L4–L5 DRG were also significantly higher (+40%) in HSVLatEnk-infected rats than in paired control polyarthritic animals ( $71.9 \pm 7.1$  vs  $49.5 \pm 5.2$  pg/mg protein, respectively; means  $\pm$  SEM;  $n = 8$  in each group;  $p < 0.01$ ). On the other hand, measurement of both substance P and CGRP concentrations in L4–L5 DRG of control polyarthritic rats ( $1.6 \pm 0.2$  and  $8.3 \pm 0.2$  ng/mg protein, respectively; means  $\pm$  SEM;  $n = 8$ ) showed comparable levels with those in HSVLatEnk-infected rats ( $1.4 \pm 0.1$  and  $8.1 \pm 0.4$  ng/mg protein;  $n = 8$ ; respectively).

Immunofluorescence investigations showed that no ME-immunoreactive neurons could be detected in L4–L5 DRG and corresponding dorsal roots of untreated polyarthritic rats (Fig. 6A,B). Furthermore, neuronal processes stained for MELM were only rarely detected in peripheral outputs of L4–L5 DRG in these animals (Fig. 6C). Infection of rats with HSVLatEnk re-





**Figure 6.** Immunofluorescent detection of Met-enkephalin-like material. Fifteen micrometer sections were stained with monoclonal anti-ME antibody (Valbiotech). No ME staining was detected in DRG neuronal cell bodies (*A*) and dorsal roots (*B*) of untreated polyarthritic rats. Only few neuronal processes stained for ME could be visualized in sciatic nerves of these animals (*C*). In HSVLatEnk-infected polyarthritic rats, numerous ME-stained neuronal soma were present in L4–L5 DRG (*D*). Scarce neuronal processes were stained in dorsal roots (*E*), whereas numerous nerve fibers positively labeled for ME were present in sciatic nerves (*F*) of HSVLatEnk-infected rats. Scale bar: *A*, *D*, 250  $\mu$ m; *B*, *C*, *E*, *F*, 70  $\mu$ m.

sulted in the appearance of numerous positively stained neuronal cell bodies in L4–L5 DRG (Fig. 6*D*). Approximately 20% of MELM-positively labeled cell bodies contained CGRP-like material or substance P-like material (data not shown). No or only scarce MELM-positively labeled nerve fibers were visualized in dorsal roots of infected animals (Fig. 6*E*). In contrast, a relatively dense bundle of neuronal processes containing MELM was observed in the peripheral output of L4–L5 DRG in HSVLatEnk-infected polyarthritic rats (Fig. 6*F*).

## DISCUSSION

Together with pain, erosive inflammation of the joints and new bone proliferation represent prominent symptoms of polyarthritis in both humans and animal models of the disease. This report shows that PA overexpression in lumbar DRG neurons of polyarthritic rats induces mainly peripherally mediated antihyperalgesic effects and reduces disease-related functional disability in these animals. Furthermore, our studies also demonstrate the beneficial effect of PA-derived peptides on hindpaw joint lesions because overproduction of these opioids limited the progression of bone erosion and periostitis.

HSV type 1-derived vectors have been successfully used for transgene delivery into sensory neurons, and we showed recently their potency to drive PA expression in rat DRG neurons (Davar et al., 1994; Antunes-Bras et al., 1998, 2001; Goins et al., 1999; Wilson et al., 1999). In our previous studies, peripheral infection of healthy rats with replication-competent vectors resulted in vector expression limited to L4–L5 DRG (Antunes-Bras et al., 1998); however, possibly because of the peculiar immune status of

polyarthritic rats, we found in a first series of experiments that, in some of these animals, recombinant vectors could spread from the DRG to the spinal cord. This observation led us to generate conditional defective TK-deleted HSV vectors, severely impaired for the acute replication in ganglionic neurons (Coen et al., 1989). To ensure long-term and relatively high levels of PA-derived peptides synthesis, we placed the rat PA cDNA sequence under the control of Lat-LTR promoter whose *in vivo* activity is well documented (Lokensgard et al., 1994; Antunes-Bras et al., 1998). To detect all viral vectors (even those that eventually did not synthesize transgene-derived mRNA), we performed *in situ* hybridization histochemistry not only with an ME mRNA complementary probe but also with a probe complementary to viral latency-associated transcripts. The results obtained with both probes clearly showed that TK-negative recombinants remained restricted to L4–L5 DRG of polyarthritic rats and did not spread to adjacent ganglia or the spinal cord, as expected for this vector with reduced replication in neurons (Coen et al., 1989). Despite the presence of numerous neurons synthesizing MELM in L4–L5 DRG 3 weeks after HSVLatEnk inoculation, only few nerve fibers endowed with MELM were visualized in L4–L5 dorsal roots. In contrast, numerous neuronal profiles containing MELM were present in sciatic nerves of HSVLatEnk-infected polyarthritic rats. These results are consistent with our previous studies in healthy rats showing that, in PA overexpressing animals (as in controls), MELM is preferentially transported to the peripheral terminals of primary afferents from which it can be released by electrical stimulation of the sciatic nerve (Antunes-Bras et al., 2001). Together, these data suggest that, in HSVLatEnk-infected polyarthritic rats, overproduced MELM was also mainly transported to the peripheral side of primary afferent fibers and presumably released.

The concentrations of substance P and CGRP in DRG of polyarthritic rats, which are increased compared those in control healthy rats (Kar et al., 1991; Smith et al., 1992), were not modified in animals infected with either HSVLat $\beta$ -gal or HSVLatEnk, suggesting that neither the infection per se nor the ME overexpression affects the DRG content in these major neuropeptides synthesized in sensory ganglia. However, opioids are known to modulate the substance P and CGRP release from primary afferent neurons (Mauborgne et al., 1987; Pohl et al., 1989). Further investigations are thus needed to examine whether their release, which is altered in polyarthritic rats (Cesselin et al., 1999), could be modified in HSVLatEnk-infected rats, notably at the periphery, at which both peptides exert proinflammatory effects.

An important finding is the reduction of hindpaw diameter associated with reduced progression of joint lesions in HSVLatEnk-infected polyarthritic rats. Interestingly, radiological examination of hindpaws revealed reduced osseous lesions and bone proliferation, particularly at the tarsus and metatarsus joints, i.e., at the paw levels at which primary afferents infected with HSVLatEnk project. Immunomodulatory action of opioids is well documented (for review, see Stefano et al., 1996). The existence of a dense network of sensory nerves innervating the periosteum, the bone marrow, and the osteochondral junction (Bjurholm et al., 1988; Hill and Elde, 1991; Hukkanen et al., 1992), as well as the presence of opioid binding sites on osteoblasts and chondrocytes (Castano et al., 1991), support the idea of a direct action of peripherally released opioids on these tissues. Our data are consistent with previous reports suggesting that opioids can modulate inflammation and attenuate joint damage in

polyarthritic rats (Levine et al., 1986; Walker et al., 1996; Binder and Walker, 1998).

Overproduction of PA-derived opioid peptides in sensory nerves also resulted in an antihyperalgesic activity. In particular, HSVLatEnk-infected rats exhibited an increased latency of foot withdrawal elicited by noxious radiant heat. However, an acute painful stimulus does not actually reflect the dimension of persistent, spontaneous pain. This is particularly true in polyarthritic rats in which the most prominent behavioral change is a marked reduction of locomotion as a result of intense pain and joints alterations. Indeed, horizontal and vertical mobility in polyarthritic rats were severely reduced (rearings being almost absent), and measurement of locomotor activity provides a sensitive evaluation of "functional disability" in these animals (Larsen and Arnt, 1985; Cain et al., 1997; Lindner et al., 1999). Of special interest in this study was the impressive amelioration of the mobility of HSVLatEnk-infected polyarthritic rats that recovered up to nearly 70% of that of healthy rats. With regard to pain-related behavior, our data are in accordance with the recently reported antihyperalgesic effects of overexpressed PA or  $\beta$ -endorphin at the spinal level in animals with experimentally induced pain (Finegold et al., 1999; Wilson et al., 1999).

The involvement of central and/or peripheral opioid receptors in both the antihyperalgesic response and the improved mobility of HSVLatEnk-infected polyarthritic rats was assessed using naloxone, a centrally and peripherally acting opioid receptor antagonist, and naloxone methiodide, an antagonist acting exclusively at the periphery. Taking into account that PA overexpression in sensory neurons of HSVLatEnk-infected rats is a continuous, long-lasting (i.e., for several weeks) process, we reasoned that prolonged delivery of opioid receptor antagonists should be a valuable approach to inhibit the effects of overproduced ME. Both naloxone and naloxone methiodide, without significant effect in control polyarthritic rats, reversed with a similar efficacy both the antihyperalgesic response and the improved mobility in HSVLatEnk-infected polyarthritic rats. This finding is in line with previous observations showing that naloxone [but at a markedly higher dose (50 mg/kg, i.p.) than that used here] antagonized the antihyperalgesic responses to capsaicin and dimethylsulfoxide as a result of overexpression of human PA in recombinant virus-infected mice (Wilson et al., 1999). In addition, the fact that naloxone methiodide was as efficient as naloxone to suppress the antihyperalgesic response further supports the idea that peripheral opioid receptors play a key role in opioid-induced reduction of inflammatory pain (Stein and Yassouridis, 1997; Binder and Walker, 1998) and related disability in polyarthritic rats infected with HSVLatEnk. However, because sparse MELM-containing nerve fibers were observed in dorsal roots of HSVLatEnk-infected rats, it cannot be excluded that spinal opioid receptors also contributed to the antihyperalgesic effects of PA overexpression in DRG neurons.

Although HSV latency is an almost exclusive feature of nervous tissue (Steiner and Kennedy, 1995), the possibility of latent infection of non-neuronal cells at the site of HSVLatEnk inoculation could not be ruled out. This possibility could account for the mainly peripherally mediated affects observed in our study and, to some extent, also in that of Wilson et al. (1999). However, these authors found that intrathecal administration of naloxone reversed the antihyperalgesic effect in mice infected with proenkephalin encoding vector, thereby suggesting that primary afferents represented the major supply of overproduced opioid peptides. Our previous results also support this idea. Indeed, we

found in normal HSVLatEnk-infected rats that MELM was released not only at the dorsal horn of the spinal cord by direct electrical stimulation of dorsal roots but also and to a greater extent at the periphery after electrical stimulation of the sciatic nerve (Antunes-Bras et al., 2001). These results suggest that increased peripheral MELM overflow triggered by stimulation of the sciatic nerve in fact involved peripheral terminals of primary afferents rather than some non-neuronal cells.

Naloxone methiodide treatment did not affect the radiological lesion score in HSVLatEnk-infected polyarthritic rats, indicating that, under such conditions, a 3 d blockade of peripheral opioid receptors was probably too short to reverse the inhibitory action of PA overexpression on the progression of the disease.

In any case, polyarthritis-associated disability in rats appears to be the consequence of both chronic pain and mechanical affection of joints. Accordingly, the long-term amelioration in polyarthritic rats infected with HSVLatEnk probably reflects a synergic action of PA-derived peptides on both aspects of the disease. Gene therapy for rheumatoid arthritis is a rapidly growing field, and several relevant approaches are currently tested in laboratory models of arthritis (Junker and Böhlein, 1999). Thus, association of proenkephalin A (and perhaps of other opioid peptides precursors) gene with other candidate genes in dicistronic vectors might open novel perspectives for the management of both pain and osseous lesions that characterize this disease.

## REFERENCES

- Antunes-Bras JM, Epstein AL, Bourgoin S, Hamon M, Cesselin F, Pohl M (1998) Herpes simplex virus1-mediated transfer of preproenkephalin A in rat dorsal root ganglia. *J Neurochem* 70:1299–1303.
- Antunes-Bras JM, Becker C, Bourgoin S, Lombard MC, Cesselin F, Hamon M, Pohl M (2001) Met-enkephalin is preferentially transported to the peripheral processes of primary afferent fibres in both normal and proenkephalin A overexpressing rats. *Neuroscience* 103:1073–1083.
- Binder W, Walker JS (1998) Effect of the peripherally selective kappa-opioid agonist, asimadoline, on adjuvant arthritis. *Br J Pharmacol* 124:647–654.
- Björholm A, Kreibergs A, Brodin E, Schultzberg M (1988) Substance P- and CGRP-immunoreactive nerves in bone. *Peptides* 9:165–171.
- Cain CK, Francis JM, Plone MA, Emerich DF, Lindner MD (1997) Pain-related disability and effects of chronic morphine in the adjuvant-induced arthritis model of chronic pain. *Physiol Behav* 62:199–205.
- Calvino B, Crepon-Bernard MO, Le Bars D (1987) Parallel clinical and behavioural studies of adjuvant-induced arthritis in the rat: possible relationship with "chronic pain." *Behav Brain Res* 24:11–29.
- Carlton SM, Coggeshall RE (1997) Immunohistochemical localization of enkephalin in peripheral sensory axons in the rat. *Neurosci Lett* 221:121–124.
- Castano MT, Freire-Garabal M, Giraldez M, Nunez NJ, Belmonte A, Couceiro J, Jorge J (1991) Autoradiographic evidence of  $^{125}$ I-beta-endorphin binding sites in the articular cartilage of the rat. *Life Sci* 49:103–105.
- Cesselin F, Montastruc JL, Gros C, Bourgoin S, Hamon M (1980) Met-enkephalin levels and opiate receptors in the spinal cord of chronic suffering rats. *Brain Res* 191:289–293.
- Cesselin F, Benoliel JJ, Bourgoin S, Collin E, Pohl M, Hamon M (1999) Spinal mechanisms of opioid analgesia. In: *Opioids in pain control: basic and clinical aspects* (Stein C, ed), pp 70–95. Cambridge, UK: Cambridge UP.
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Coen DM, Kosz-Vnenchak MN, Jacobson JG, Leib DA, Bogard CL, Schaffer PA, Tyler KL, Knipe DM (1989) Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc Natl Acad Sci USA* 86:4736–4740.
- Colpaert FC (1987) Evidence that adjuvant arthritis in the rat is associated with chronic pain. *Pain* 28:201–222.
- Davar G, Kramer MF, Garber D, Roca AL, Andersen JK, Bebrin W, Coen DM, Kosz-Vnenchak M, Knipe DM, Breakefield XO, Isaacson O (1994) Comparative efficacy of expression of genes delivered to mouse sensory neurons with herpes virus vectors. *J Comp Neurol* 339:3–11.
- De Castro Costa M, De Sutter P, Gybels J, Van Hees J (1981) Adjuvant-

- induced arthritis in rats: a possible animal model of chronic pain. *Pain* 10:173-185.
- El Hassan AM, Lindgren JU, Hultenby K, Bergstrom J, Adem A (1998) Methionine-enkephalin in bone and joint tissues. *J Bone Miner Res* 13:88-95.
- Finegold AA, Mannes AJ, Iadarola MJ (1999) A paracrine paradigm for *in vivo* gene therapy in the central nervous system: treatment of chronic pain. *Hum Gene Ther* 10:1251-1257.
- Galbraith JA, Mrosko BJ, Myers RR (1993) A system to measure thermal nociception. *J Neurosci Methods* 49:63-68.
- Geller AI, Breakefield XO (1988) A defective HSV-1 vector expresses *Escherichia coli*  $\beta$ -galactosidase in cultured peripheral neurons. *Science* 241:1667-1669.
- Goins WF, Lee KA, Cavalcoli JD, O'Malley ME, DeKosky ST, Fink DJ, Glorioso JC (1999) Herpes simplex virus type 1 vector-mediated expression of nerve growth factor protects dorsal root ganglion neurons from peroxide toxicity. *J Virol* 73:519-532.
- Gouret C, Mocquet G, Raynaud G (1976) Use of Freund's adjuvant arthritis test in anti-inflammatory drug screening in the rat: value of animal selection and preparation at the breeding center. *Lab Animal Sci* 26:281-287.
- Harris ED (1990) Rheumatoid arthritis. Pathophysiology and implications for therapy. *N Engl J Med* 322:1277-1289.
- Hill EL, Elde R (1991) Distribution of CGRP, VIP, D beta H, SP, and NPY-immunoreactive nerves in the periosteum of the rat. *Cell Tissue Res* 264:469-480.
- Houghton AK, Valdez JG, Westlund KN (1998) Peripheral morphine administration blocks the development of hyperalgesia and allodynia after bone damage in the rat. *Anesthesiology* 89:190-201.
- Hukkanen M, Kontinen YT, Rees RG, Santavirta S, Terenghi G, Polak JM (1992) Distribution of nerve endings and sensory neuropeptides in rat synovium, meniscus and bone. *Int J Tissue React* 14:1-10.
- Junker U, Böhnlein E (1999) Gene therapy for arthritis 1996-1999. *Exp Opin Ther Patents* 9:1491-1498.
- Kar S, Gibson SJ, Rees RG, Jura WG, Brewerton DA, Polak JM (1991) Increased calcitonin gene-related peptide (CGRP), substance P, and enkephalin immunoreactivities in dorsal spinal cord and loss of CGRP-immunoreactive motoneurons in arthritic rats depend on intact peripheral nerve supply. *J Mol Neurosci* 3:7-18.
- Larsen JJ, Arnt J (1985) Reduction in locomotor activity of arthritic rats as parameter for chronic pain: effect of morphine, acetylsalicylic acid and citalopram. *Acta Pharmacol Toxicol* 57:345-351.
- Levine JD, Dardick SJ, Roizen MF, Helms C, Basbaum AI (1986) Contribution of sensory afferent and sympathetic efferents to joint injury in experimental arthritis. *J Neurosci* 6:3423-3429.
- Lindner MD, Plone MA, Francis JM, Cain CK (1999) Chronic morphine reduces pain-related disability in a rodent model of chronic, inflammatory pain. *Exp Clin Psychopharmacol* 7:187-197.
- Lokensgard JR, Bloom DC, Dobson AT, Feldman LT (1994) Long-term promoter activity during herpes simplex virus latency. *J Virol* 68:7148-7158.
- Mauborgne A, Lutz O, Legrand JC, Hamon M, Cesselin F (1987) Opposite effects of  $\mu$  and  $\delta$  opioid receptor agonists on the *in vitro* release of substance P-like material from the rat spinal cord. *J Neurochem* 48:529-537.
- Pearson CM (1956) Development of arthritis, periarthritis and periostitis in rats given adjuvant. *Proc Soc Exp Biol* 91:95-101.
- Pohl M, Lombard MC, Bourgoin S, Carayon A, Benoliel JJ, Mauborgne A, Besson JM, Hamon M, Cesselin F (1989) Opioid control of the *in vitro* release of calcitonin gene-related peptide from primary afferent fibres projecting in the rat cervical cord. *Neuropeptides* 14:151-159.
- Pohl M, Benoliel JJ, Bourgoin S, Lombard MC, Mauborgne A, Taquet H, Carayon A, Besson MJ, Cesselin F, Hamon M (1990) Regional distribution of calcitonin gene-related peptide-, substance P-, cholecystokinin-, met-enkephalin-, and dynorphin A(1-8)-like materials in the spinal cord and dorsal root ganglia of adult rats: effects of dorsal rhizotomy and neonatal capsaicin. *J Neurochem* 55:1122-1130.
- Pohl M, Collin E, Bourgoin S, Conrath M, Benoliel JJ, Nevo I, Hamon M, Giraud P, Cesselin F (1994) Expression of preproenkephalin A gene and presence of met-enkephalin in dorsal root ganglia of the adult rat. *J Neurochem* 63:1226-1234.
- Pohl M, Ballet S, Collin E, Mauborgne A, Bourgoin S, Benoliel JJ, Hamon M, Cesselin F (1997) Enkephalinergic and dynorphinergic neurons in the spinal cord and dorsal root ganglia of the polyarthritic rat: *in vivo* release and cDNA hybridization studies. *Brain Res* 749:18-28.
- Schäfer M, Carter L, Stein C (1994) Interleukin  $1\beta$  and corticotropin-releasing factor inhibit pain by releasing opioids from immune cells in inflamed tissue. *Proc Natl Acad Sci USA* 91:4219-4223.
- Smith GD, Harmar AJ, McQueen DS, Seckl JR (1992) Increase in substance P and CGRP, but not somatostatin content of innervating dorsal root ganglia in adjuvant monoarthritis in the rat. *Neurosci Lett* 137:257-260.
- Smith RL, Geller AI, Ecudero KW, Wilcox CL (1995) Long-term expression in sensory neurons in tissue culture from herpes simplex virus type 1 (HSV-1) promoters in an HSV-1-derived vector. *J Virol* 69:4593-4599.
- Stefano GB, Scharrer B, Smith EM, Hughes Jr TK, Magazine HI, Bilfinger TV, Hartman AR, Fricchione GL, Liu Y, Makman MH (1996) Opioid and opiate immunoregulatory processes. *Crit Rev Immunol* 16:109-144.
- Stein C, Yassouridis A (1997) Peripheral morphine analgesia. *Pain* 71:119-121.
- Steiner I, Kennedy PG (1995) Herpes simplex virus latent infection in the nervous system. *J Neurovirol* 1:19-29.
- Wagner EK, Devi-Rao GB, Feldman LT, Dobson AT, Zhang YK, Flangan WM, Stevens JG (1988) Physical characterization of the herpes simplex virus latency-associated transcript in neurons. *J Virol* 62:1194-1202.
- Walker JS, Chandler AK, Wilson JL, Binder W, Day RO (1996) Effect of mu-opioids morphine and buprenorphine on the development of adjuvant arthritis in rats. *Inflamm Res* 45:557-563.
- Wilson SP, Yeomans DC, Bender MA, Lu Y, Goins WF, Glorioso JC (1999) Antihyperalgesic effects of infection with a preproenkephalin-encoding herpes virus. *Proc Natl Acad Sci USA* 96:3211-3216.
- Yoshikawa K, Williams C, Sabol SL (1984) Rat brain preproenkephalin mRNA. cDNA cloning, primary structure, and distribution in the central nervous system. *J Biol Chem* 259:14301-14308.



## RESEARCH ARTICLE

# Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion

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Endogenous opiate peptides acting pre- and post-synaptically in the dorsal horn of spinal cord inhibit transmission of nociceptive stimuli. We transfected neurons of the dorsal root ganglion *in vivo* by footpad inoculation with 30  $\mu$ l ( $3 \times 10^7$  p.f.u.) of a replication-incompetent (ICP4-deleted) herpes simplex virus (HSV) vector with a cassette containing a portion of the human proenkephalin gene coding for 5 met- and 1 leu-enkephalin molecules under the control of the human cytomegalovirus immediate-early promoter (HCMV IEp) inserted in the HSV thymidine kinase (tk) locus. Vector-directed expression of enkephalin produced a significant

antinociceptive effect measured by the formalin footpad test, that was most prominent in the delayed ('tonic') phase 20–70 min after the administration of formalin. The magnitude of the antinociceptive effect diminished over 4 weeks after transduction, but reinoculation of the vector reestablished the analgesic effect, without evidence for the development of tolerance. The antinociceptive effect was blocked completely by intrathecal naltrexone. These results suggest that gene therapy with an enkephalin-producing herpes-based vector may prove useful in the treatment of pain. Gene Therapy (2001) 8, 551–556.

**Keywords:** HSV; pain; enkephalin; gene transfer

## Introduction

The pharmacologic activity of opiate drugs corresponds to the distribution of endogenous opioid peptides and their receptors in the nervous system.<sup>1,2</sup> One important location for the action of opioids is the dorsal horn of spinal cord where opioid receptors are found on both spinal interneurons and on the terminals of the primary afferent nociceptors whose cell bodies are in the dorsal root ganglia (DRG).<sup>3,4</sup> *In vitro* opioids produce a direct inhibitory effect on the electrical activity of spinothalamic projection neurons of dorsal horn or trigeminal nucleus,<sup>5–7</sup> and act presynaptically to inhibit release of neurotransmitter from DRG neurons.<sup>8–11</sup> Intrathecal administration of morphine inhibits the release of substance P by the primary afferent nociceptor *in vivo*,<sup>12</sup> which correlates with the efficacy of intrathecal opiates that are used in clinical applications.

More than 20 endogenous opioid peptides have been identified, most of which share the N-terminal sequence of Tyr-Gly-Gly-Phe-Met or Tyr-Gly-Gly-Phe-Leu; they are produced as the cleavage products of three distinct precursors that have been termed proenkephalin A, prodynorphin, and proopiomelanocortin.<sup>13,14</sup> Proenkephalin A is the only opioid peptide precursor in spinal cord, where the gene is expressed primarily in interneurons in

the dorsal horn and the product cleaved to produce met- and leu-enkephalin. It is presumed that the pre- and post-synaptic anti-nociceptive effects of opiate drugs described above result from activation of the same opioid receptors that respond to endogenous production and release of met- and leu-enkephalin.

Gene transfer represents a novel means to express identified transgenes in specific locations in the nervous system, and herpes simplex virus-based vectors have a special utility for primary sensory neurons. Wild-type HSV is a neurotropic double-stranded DNA virus that, following primary infection of epithelium, is carried by retrograde axonal transport to the dorsal root ganglion (DRG) where the viral genome may establish a life-long latent state.<sup>15–17</sup> Because HSV genes involved in viral replication are expressed in a rigid temporal cascade, deletion of essential immediate-early (IE) genes from the HSV genome allows the creation of a vector that is incapable of replicating in normal tissue *in vivo*, but nonetheless is capable of the efficient establishment of a quiescent state that is similar to natural viral latency but without the potential for reactivation.<sup>18,19</sup> We have previously demonstrated that genomic HSV-based vectors can be employed to express biologically active nerve growth factor<sup>20</sup> and the anti-apoptotic peptide Bcl-2<sup>21</sup> *in vitro* and *in vivo*.

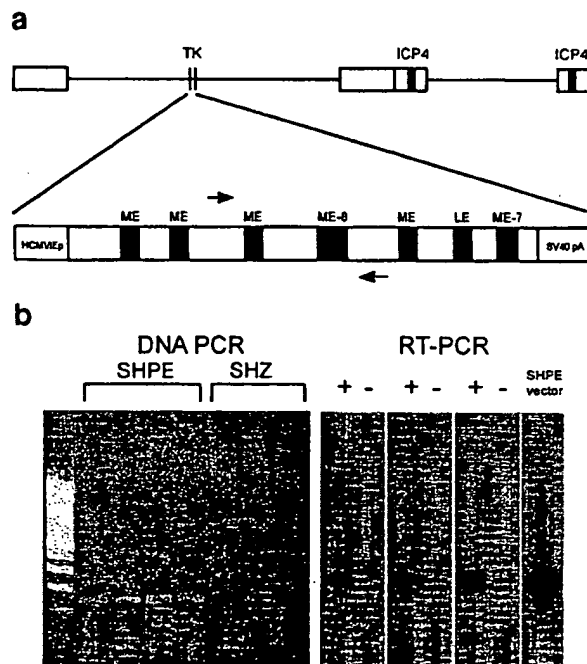
In the experiments described in this report, we tested the ability of a genomic HSV vector expressing enkephalin to produce an antinociceptive effect in rats. We constructed a replication-incompetent genomic HSV vector deleted for the essential IE gene ICP4, with the proen-

kephalin coding sequence under the control of the human cytomegalovirus immediate-early promoter (HCMV IEp) element in the viral tk locus, and used that vector to transfect neurons of the rat lumbar DRG by foot-pad inoculation. We demonstrate that this vector produces an antinociceptive effect, significantly reducing nociceptive behavior in the delayed 'tonic' phase after the subcutaneous administration of formalin.

## Results

Inoculation of the footpad with the non-replicating vector SHPE resulted in transduction of lumbar DRG and expression of proenkephalin mRNA. Using primers specific for the human proenkephalin sequence to amplify a 248 bp fragment of the human gene insert (Figure 1a), we found specific bands from all four SHPE-injected rats killed 1 week after inoculation (Figure 1b, left). No human proenkephalin DNA was found in DRG of rats injected with the *lacZ*-containing control vector SHZ (Figure 1b, left). Evaluation of DRG from a second group of SHPE-injected animals for transgene expression by RT-PCR demonstrated the presence of human proenkephalin RNA at the same time-point (Figure 1b, right).

Expression of the human peptide in transfected neurons was assessed *in vitro*. Mouse DRG neurons transfected with SHPE expressed human PE detected by immunocytochemistry, while DRG neurons transfected with the control SHZ vector showed only background immunofluorescence similar to uninfected neurons



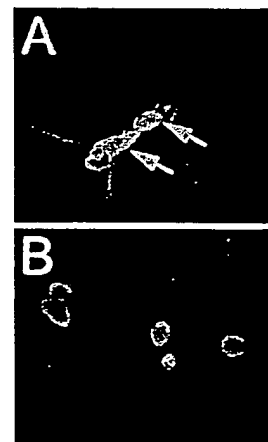
**Figure 1** (a) Schematic representation of the HSV vector genome, with insertion of the proenkephalin gene in the tk locus. Arrows represent location of primers used for PCR analysis. (b) PCR and RT-PCR from transduced DRG 1 week after inoculation demonstrates the presence of the human proenkephalin sequences in SHPE but not control SHZ infected DRG (left panel), and the presence of human proenkephalin RNA in SHPE infected ganglia (right panel; + indicates samples amplified with reverse transcriptase, - indicates samples amplified without reverse transcriptase).

(Figure 2). We were not able to identify the peptide *in vivo* by immunofluorescence (data not shown).

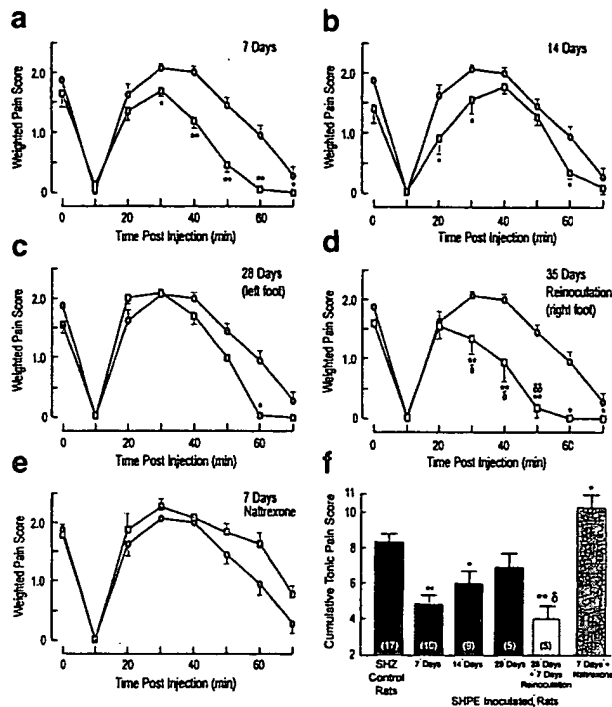
Injection of dilute formalin subcutaneously into the footpad of control animals caused the rats to exhibit nociceptive behaviors as previously described,<sup>22</sup> with an immediate pain response (at time 0), a period of normal behavior (observed at 10 min after formalin injection), followed by a characteristic increasing and then decreasing pain response lasting over 60 min (Figure 3a, control curve - filled circles). Rats injected with SHPE 1 week before formalin testing showed a significant reduction in nociceptive behaviors at 30, 40, 50, 60, and 70 min after formalin injection (Figure 3a, black squares). The antinociceptive effect of the vector was reversed by an intrathecal injection of naltrexone (20 nmol) administered 5 min before the injection of formalin (Figure 3e, gray squares). Intraperitoneal injection of naloxone (50 mg/kg) also reversed the effect of the vector (data not shown). Because injection of formalin may cause long-lasting alterations to the subcutaneous tissue each animal was tested only once, and the naltrexone experiments were performed on a different set of animals than the 1 week test group.

Rats inoculated with SHPE 2 weeks before formalin testing showed a less pronounced antinociceptive effect that was statistically significant only at 20, 30 and 60 min after formalin (Figure 3b, black squares). The reduction in response to the vector is consistent with previous observations regarding the time-course of transgene expression by HSV vectors utilizing the HCMV IEp to drive transgene expression.<sup>23,24</sup>

Rats injected with SHPE 4 weeks before formalin testing showed a quantitatively smaller response to the vector, with statistically significant differences only at 60 min after formalin (Figure 3c, black squares). We had anticipated that the response to vector transduction would be severely attenuated by 28 days, and for that reason had inoculated those animals in both the left and right rear footpads at the time of initial vector inoculation. After formalin testing in the left footpad, as described above



**Figure 2** Immunofluorescent detection of human PE in DRG neurons transfected with SHPE *in vitro* (A). Neurons transfected with the *lacZ*-expressing control vector SHZ demonstrate only background immunofluorescence (B). The photomicrographs shown were taken with identical exposures, chosen to show the control vector-infected cells (B) which were identical to uninfected neurons (data not shown).



**Figure 3** Weighted pain score following injection of dilute formalin in SHZ inoculated control animals (circles) and SHPE inoculated animals (squares) at 7 days (a), 14 days (b), 28 days (c), and 35 days in animals reinoculated 7 days before testing (d). Intrathecal naltrexone reversed the antinociceptive effect in animals tested 7 days after SHPE inoculation (e). A cumulative pain score, representing the sum of the weighted pain scores from 20 to 70 min, demonstrates the time-course of the antinociceptive effect of the vector (f). The number of animals in each group is shown in parentheses. The same control group is shown in all graphs.

and shown in Figure 3c, we reinoculated the same rats in the right footpad with SHPE (identical to the initial inoculation), and retested the animals with an injection of formalin into the right footpad 1 week later (35 days after the initial vector inoculation, 7 days after reinoculation). Formalin testing in the right (reinoculated) footpad at 35 days showed that a robust antinociceptive response, which was statistically significant at 30, 40, 50, 60 and 70 min after formalin, had been reestablished (Figure 3d, open squares). Separate control animals (those inoculated with the SHZ vector) were tested at the same time as SHPE inoculated animals at each time-point, however, all control animals were grouped together for statistical comparison to SHPE inoculated animals, and that control curve is reproduced in each panel of Figure 3a–e (black circles).

The relative reduction in nocifensive behavior at the several different times after vector inoculation is shown in Figure 3f, where we have plotted a cumulative pain score representing the sum of the scores at the 20 to 70 min intervals for each of the times after vector inoculation. The cumulative pain score illustrates the significant reduction in nocifensive behavior at 7 days after vector inoculation, the reduction in that effect in animals inoculated 2 or 4 weeks before testing, and the reestablishment of the response by reinoculation.

## Discussion

The data presented in this report demonstrate that a non-replicating genomic HSV-based vector engineered to express proenkephalin can be used to transduce primary sensory neurons of the DRG to produce an analgesic effect in rats. The formalin test<sup>22</sup> allows the observation of nocifensive behaviors in unrestrained animals over a prolonged period of time. Human subjects describe the pain caused by formalin injection as an initially intense, sharp or stinging sensation that gives way to a steady throbbing ache that gradually disappears over 1 h.<sup>22</sup> The response recorded in the 'tonic' phase in animals corresponds to that steady throbbing ache, in contrast to acute pain sensation.

This report substantially extends the observations of Wilson and colleagues<sup>25</sup> who used a replicating herpes vector, KHPE, to deliver the same gene product to the DRG by subcutaneous injection into the dorsum of the foot in mice. While no primary antinociceptive effect of the vector was observed in those experiments, enkephalin production by the vector resulted in an antihyperalgesic effect that was demonstrated by an increased latency to withdraw the foot from noxious heat after sensitization of C-fibers by application of capsaicin, or sensitization of A $\delta$  fibers by application of dimethyl sulfoxide.<sup>25</sup> Foot withdrawal to noxious radiant heat before sensitization is an acute pain response which in its time-course correlates with the nociceptive behavior in the formalin test immediately after injection of formalin. In agreement with Wilson's study, the response to formalin immediately after injection was not altered by inoculation with vector SHPE in our experiments. The antinociceptive effect we observed in the tonic phase after formalin injection following inoculation with SHPE represents an analgesic effect in a well-established model of inflammatory pain and extends the potential utility of gene transfer for treatment of pain.

The second major difference between these studies and the previous report is the nature of the vector. KHPE<sup>25</sup> is a replication-competent herpes virus that presumably replicates in epithelial cells following inoculation and before axonal transport. SHPE (the current report) is a replication-incompetent mutant that despite not dividing in the epithelium nonetheless efficiently transfects the DRG. This is an important consideration for potential therapeutic application. The difference between their use of a replicating HSV vector and our use of a non-replicating vector may be one explanation for the fact that they were able to detect the gene product by immunocytochemistry *in vivo*, whereas we were unable to do so. Unfortunately, the antibody they used for their studies is no longer available, so that direct comparison of their immunochemical results with our results is not possible. Nonetheless, using a similar antibody specific for the human peptide, we have demonstrated expression of the peptide product in transfected cells *in vitro*, and the RT-PCR data provides specific evidence in our studies for expression of the human transgene in transfected DRG neurons *in vivo*. We found that the antinociceptive effect was maximal at 1 week after inoculation. This time-course is also different than the time course reported by Wilson *et al*. We believe it is likely that the difference in time-course is due to differences in the models tested, as in preliminary studies we have found a different time-

course of antinociceptive effect of the same vector in a model of neuropathic pain (data not shown).

These experiments differ substantially from other gene transfer approaches that have been reported previously. An adenoviral vector expressing the  $\beta$ -endorphin coding sequence infused intrathecally in rats has been reported to attenuate the hyperalgesic response in the carageenan model of inflammatory pain<sup>26</sup> but had no effect on the latency to withdraw a non-inflamed paw from radiant thermal heat. Transgene expression from the adenoviral vector appeared to be limited to the pia mater, with release of  $\beta$ -endorphin into the cerebrospinal fluid.<sup>26</sup> In contrast, HSV-mediated gene transfer results in specific transfection of primary sensory neurons in the DRG. The mechanism of adenoviral gene transfer is more like cell transplantation studies which have been reported. For example, retrovirus vectors have been used to transfect and express  $\beta$ -endorphin in fibroblasts,<sup>27</sup> and spinal transplantation of adrenal medullary cells which naturally release opioid peptides reduces nociceptive behaviors (tail flick and paw pinch response)<sup>28</sup> and have been reported to decrease experienced pain in humans with pain and terminal cancer;<sup>29</sup> similar results have been demonstrated using the AtT-20 pituitary tumor-derived cell line modified to constitutively secrete enkephalin.<sup>30,31</sup> However, these approaches all rely on release of peptides into CSF, rather than localized release of enkephalin in the dorsal horn.

We presume that release of the HSV-mediated opiate peptides occurs from the central terminal of primary afferent neurons in the dorsal horn of spinal cord. While we have not identified the specific neurons that harbor the latent HSV genomes, there is no reason to expect that the vector should show a preference for nociceptors. Because A $\delta$  and C fibers typically constitute 70–80% of nerve fibers innervating the skin<sup>13</sup> it is not unlikely that a substantial proportion of the transduced neurons project to the superficial layers of dorsal horn. Even release of transgene-coded enkephalin by non-nociceptive afferents would release the peptide in the near vicinity of the second order neuron in the spinal cord. We have previously demonstrated *in vitro* that an HSV vector transgene-coded peptide (NGF) is appropriately processed and released from neurons *in vitro*.<sup>20</sup> The affinity of enkephalins for opiate receptors is roughly half that of morphine, yet following direct injection *in vivo*, met-enkephalin is more than one order of magnitude weaker than morphine as an analgesic,<sup>32</sup> and the analgesic effect of the peptide is short-lived;<sup>33</sup> both of these phenomena result from the distribution of an endogenous neutral protease 'enkephalinase'<sup>34</sup> in the same distribution as endogenous opiates and their receptors. One potential advantage of gene transfer to primary sensory neurons is that continuous local release of an opiate peptide in the spinal cord may avoid some of the problems that result from the central actions of systemic or intrathecal administration of opiate drugs.<sup>35</sup>

Is it possible that the biological effect of released vector-encoded enkephalin is mediated through actions at peripheral, rather than the central terminal of the sensory neuron?<sup>36</sup> Mu, delta and kappa opioid receptors have been demonstrated on the peripheral terminals of sensory axons,<sup>37,38</sup> and the peripheral injection of opioid drugs relieves inflammatory pain in animal models.<sup>38,39</sup> However, intrathecal injection of naltrexone completely

reversed the analgesia produced by the vector supporting the interpretation that the analgesic effect occurs at the level of the spinal cord rather than peripherally. In fact, animals that received naltrexone demonstrated a significantly higher cumulative tonic pain score than control animals, which may have resulted from block of endogenous opioid effects in addition to block of vector-expressed enkephalin. We also have no direct evidence regarding the development of tolerance in this model. However, the loss of effect over several weeks parallels the known time-course of expression of transgenes from the HSV vector in neurons using the HCMV promoter,<sup>17,23</sup> and the reestablishment of an antinociceptive effect by reinoculation also suggests that tolerance had not developed.

There are several potential avenues of investigation that may be developed from the observations reported here. We do not know if the antinociceptive effect of the enkephalin vector is specific for inflammatory pain, of the sort modeled by the formalin test, or whether similar effects can be achieved in models of neuropathic pain or pain caused by cancer. We will need to determine if the duration of the analgesic effect can be extended by employing a vector that uses the HSV latency-active promoter element to drive enkephalin expression.<sup>40</sup> It will also be of interest to determine whether coexpression of other gene products from the same vector may improve the analgesic potency of the vector. However, the results of the current experiments, demonstrating that a non-replicating genomic HSV-based vector expressing proenkephalin provides an antinociceptive effect in rats, is an important proof of principle demonstration of a novel analgesic therapeutic modality.

## Materials and methods

### Vector construction

The vector SHPE (Figure 1a) was constructed as previously described.<sup>25</sup> A cassette containing the HCMV IEp, SV40 intron, human proenkephalin cDNA and SV40 polyadenylation sequence<sup>41</sup> was cloned into a plasmid (pBluescript II SK; Stratagene, La Jolla, CA, USA) containing a lox P site, and inserted into the tk locus of the ICP4-deleted HSV vector d120k-lox, a modified version of d120<sup>42</sup> that was previously engineered to contain a loxP site at the tk locus,<sup>43</sup> by cre-lox recombination.<sup>44</sup> This vector contains only the proenkephalin PE transgene, without other reporter transgenes. The control vector SHZ<sup>43</sup> was identical to SHPE except that the inserted cassette contained the *E. coli lacZ* reporter gene under the control of the HCMV IEp in the tk locus. Both the PE and control vector were isolated by three rounds of limiting dilution, and the final vector purified at  $1 \times 10^9$  p.f.u./ml.

### Immunocytochemical determination of enkephalin expression

DRG from 17-day rat embryos were dissociated with 0.25% trypsin-1 mM EDTA for 30 min at 37°C with constant shaking and then plated on poly-D-lysine-coated coverslips at  $10^5$  cells per well in a 24-well plate in 500  $\mu$ l of defined Neurobasal media supplemented with  $1 \times B027$ ,  $1 \times$  GlutaMAX, 0.5  $\mu$ g AlbuMAX II (Gibco-BRL, Rockville, MD, USA) per ml, 100 U of penicillin per ml, and 100 ng of 7.0s NGF per ml (Sigma, St Louis, MD, USA).



Fourteen days after plating the cells were infected with either vector SHPE or vector SHZ at a multiplicity of infection (MOI) of 1 for 1 h, washed in fresh media, and incubated overnight. The cells were then washed and placed in fresh media containing 1 mM colchicine (Sigma) and incubated for 2 h, after which they were fixed in Histochoice (Amresco, Solon, OH, USA) for 20 min at room temperature. Enkephalin, produced by vector SHPE, was detected using an anti-enkephalin primary antibody (Pennisula Laboratories, Belmont, CA, USA) at 1:200 in PBS containing 1% goat serum, incubated overnight at 4°C and visualized using a Cy3-conjugated anti-rabbit secondary antibody (Sigma).

#### Vector inoculation

Male Wistar rats (Charles River, Boston, MA, USA) weighing 225–250 g were used for these experiments. The animals were anesthetized with chloral hydrate (400 mg/kg) and 30 µl containing  $3 \times 10^6$  p.f.u. of either SHPE or SHZ was injected subcutaneously under the skin on the plantar aspect of one or both rear paws.

#### Tissue analysis of transgene expression

One week after vector inoculation, a subset of rats was killed, the DRGs were removed, frozen on dry ice, and stored at –80°C. DNA was isolated from the DRGs of four SHPE and three SHZ injected rats using a Qiagen kit according to the manufacturer's instructions (Valencia, CA, USA). A 248 bp fragment of human PE was amplified (94°C for 45 s, 63°C for 45 s, 72°C for 1.5 min, 40 cycles) using the primers 5'-AGAGGCCAATGG AAGTGAGA-3' (forward) and 5'-CAGCTCTTTGG CTTTCATCT-3' (reverse), a RoboCycler (Stratagene), and Perkin-Elmer (Foster City, CA, USA) Amplitaq Gold enzyme (20 µl total reaction volume, consisting of 0.2 mM dNTPs, 10 pM primers, and 0.5 unit of Taq polymerase). The PCR products were separated on a 1.5% agarose gel containing ethidium bromide.

Total RNA was extracted from the DRGs of three SHPE transfected rats using TriReagent (Sigma). Approximately 1 µg of DNaseI-treated or untreated total RNA was used to synthesize cDNA using a GeneAmp Kit (Perkin Elmer). A 248 bp fragment of human PE was amplified as described above except that the reaction mixture contained 0.67 µM  $^{32}$ P-dCTP (3000 Ci/mmol), the products separated on a 6% polyacrylamide gel, and visualized by autoradiography using Kodak (New Haven, CT, USA) X-OMAT film.

#### Formalin test

Nociception was evaluated by quantitation of nocifensive behavior using the formalin footpad test.<sup>22</sup> A dilute solution of formalin (2.5%) was injected subcutaneously in the plantar aspect of the same foot that had received the viral inoculation, and the rats then placed into a 48 × 27 × 20 cm plastic box positioned over a mirror tilted at a 45° angle. Beginning 30 s after the injection of formalin, and once every 10 min thereafter, nocifensive behaviors were recorded by a blinded observer for 3 min; a weighted pain score was derived based on the amount of time the animal exhibited each behavior during the 3 min period of observation.<sup>22</sup> The weighted pain score was plotted against time, and compared between control and treatment groups using a one-way ANOVA. Each animal was tested only once with formalin. There were

17 animals in the control group, 10 animals in the group tested 1 week after vector inoculation, nine animals in the group tested 2 weeks after vector inoculation, five animals in the group tested at 4 weeks and five animals in the group that was reinoculated at 4 weeks and tested 1 week later. Three animals were tested with intrathecal naltrexone.

#### Intrathecal injection

The site of action of HSV-mediated analgesia was assessed through intrathecal injection of naltrexone in rats inoculated with either SHPE or SHZ. Seven days following vector inoculation, rats were injected with 20 nmol of naltrexone (Sigma) in sterile 0.9% saline via direct transcutaneous injection between the dorsal aspects of L5 and L6.<sup>45</sup> Five minutes later, nocifensive behavior was evaluated using the formalin test.

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#### References

- 1 Pasternak GW. Pharmacological mechanisms of opioid analgesics. *Clin Neuropharmacol* 1993; 16: 1–18.
- 2 Olson GA, Olson RD, Vaccarino AL, Kastin AJ. Endogenous opiates: 1997. *Peptides* 1998; 19: 1791–1843.
- 3 Yaksh TL. Pharmacology and mechanisms of opioid analgesic activity. *Acta Anaesthesiol Scand* 1997; 41: 94–111.
- 4 Pohl M et al. Enkephalinergic and dynorphinergic neurons in the spinal cord and dorsal root ganglia of the polyarthritic rat – *in vivo* release and cDNA hybridization studies. *Brain Res* 1997; 749: 18–28.
- 5 Jones SL, Sedivec MJ, Light AR. Effects of iontophoresed opioids on physiologically characterized laminae I and II dorsal horn neurons in the cat spinal cord. *Brain Res* 1990; 532: 160–174.
- 6 Grudt TJ, Williams JT. mu-Opioid agonists inhibit spinal trigeminal substantia gelatinosa neurons in guinea pig and rat. *J Neurosci* 1994; 14: 1646–1654.
- 7 Willcockson WS et al. Actions of opioids on primate spinothalamic tract neurons. *J Neurosci* 1986; 6: 2509–2520.
- 8 Macdonald RL, Nelson PG. Specific-opiate-induced depression of transmitter release from dorsal root ganglion cells in culture. *Science* 1978; 199: 1449–1451.
- 9 Werz MA, Grega DS, MacDonald RL. Actions of mu, delta and kappa opioid agonists and antagonists on mouse primary afferent neurons in culture. *J Pharmacol Exp Ther* 1987; 243: 258–263.
- 10 Jęftinija S. Enkephalins modulate excitatory synaptic transmission in the superficial dorsal horn by acting at mu-opioid receptor sites. *Brain Res* 1988; 460: 260–268.
- 11 Pohl M et al. Opioid control of the *in vitro* release of calcitonin gene-related peptide from primary afferent fibres projecting in the rat cervical cord. *Neuropeptides* 1989; 14: 151–159.
- 12 Yaksh TL et al. Intrathecal morphine inhibits substance P release from mammalian spinal cord *in vivo*. *Nature* 1980; 286: 155–157.
- 13 Millan MJ. The induction of pain: an integrative review. *Prog Neurobiol* 1999; 57: 1–164.
- 14 Cesselin F. Opioid and anti-opioid peptides. *Fundam Clin Pharmacol* 1995; 9: 409–433.
- 15 Ward Y et al. Control of MAP kinase activation by the mitogen-induced threonine/tyrosine phosphatase PAC1. *Nature* 1994; 367: 651–657.



- 16 Roizman B, Sears A. Herpes simplex viruses and their replication. In: Fields BN et al. (eds). *Fields Virology*. Lippincott-Raven: Philadelphia, 1996, pp 2231-2295.
- 17 Fink DJ, DeLuca NA, Glorioso JC. Gene transfer to neurons using herpes simplex virus-based vectors. *Ann Rev Neurosci* 1996; 19: 265-287.
- 18 Krisky DM et al. Deletion of multiple immediate-early genes from herpes simplex virus reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Therapy* 1998; 5: 1593-1603.
- 19 Wolfe D et al. Engineering herpes simplex virus vectors for CNS applications. *Exp Neurol* 1999; 159: 34-46.
- 20 Goins WF et al. Herpes simplex virus type 1 vector-mediated expression of nerve growth factor protects dorsal root ganglion neurons from peroxide toxicity. *J Virol* 1999; 73: 519-532.
- 21 Yamada M et al. Herpes simplex virus vector-mediated expression of Bcl-2 prevents 6-hydroxydopamine-induced degeneration of neurons in the substantia nigra *in vivo*. *Proc Natl Acad Sci USA* 1999; 96: 4078-4083.
- 22 Dubuisson D, Dennis SG. The formalin test: a quantitative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 1977; 4: 161-174.
- 23 Fink DJ, Glorioso JC. Herpes simplex virus-based vectors: problems and some solutions. *Adv Neurol* 1997; 72: 149-156.
- 24 Fink DJ, DeLuca NA, Goins WF, Glorioso JC. Gene transfer to neurons using herpes simplex virus-based vectors. *Annu Rev Neurosci* 1996; 19: 265-287.
- 25 Wilson SP et al. Antihyperalgesic effects of infection with a preproenkephalin-encoding herpes virus. *Proc Natl Acad Sci USA* 1999; 96: 3211-3216.
- 26 Finegold AA, Mannes AJ, Iadarola MJ. A paracrine paradigm for *in vivo* gene therapy in the central nervous system: treatment of chronic pain. *Hum Gene Ther* 1999; 10: 1251-1257.
- 27 Beutler AS et al. Retrovirus-mediated expression of an artificial beta-endorphin precursor in primary fibroblasts. *J Neurochem* 1995; 64: 475-481.
- 28 Wang H, Sagen J. Absence of appreciable tolerance and morphine cross-tolerance in rats with adrenal medullary transplants in the spinal cord. *Neuropharmacology* 1994; 33: 681-692.
- 29 Winnie AP et al. Subarachnoid adrenal medullary transplants for terminal cancer pain. A report of preliminary studies (see comments). *Anesthesiology* 1993; 79: 644-653.
- 30 Wu HH, McLoon SC, Wilcox GL. Antinociception following implantation of AtT-20 and genetically modified AtT-20/hENK cells in rat spinal cord. *J Neural Transplant Plast* 1993; 4: 15-26.
- 31 Wu HH, Wilcox GL, McLoon SC. Implantation of AtT-20 or genetically modified AtT-20/hENK cells in mouse spinal cord induced antinociception and opioid tolerance. *J Neurosci* 1994; 14: 4806-4814.
- 32 Chang JK, Fong BT, Pert A, Pert CB. Opiate receptor affinities and behavioral effects of enkephalin: structure-activity relationship of ten synthetic peptide analogues. *Life Sci* 1976; 18: 1473-1481.
- 33 Belluzzi JD et al. Analgesia induced *in vivo* by central administration of enkephalin in rat. *Nature* 1976; 260: 625-626.
- 34 Waksman G, Hamel E, Fournie-Zaluski MC, Roques BP. Autoradiographic comparison of the distribution of the neutral endopeptidase 'enkephalinase' and of mu and delta opioid receptors in rat brain. *Proc Natl Acad Sci USA* 1986; 83: 1523-1527.
- 35 Schug SA, Zech D, Grond S. Adverse effects of systemic opioid analgesics. *Drug Saf* 1992; 7: 200-213.
- 36 Carlton SM, Coggeshall RE. Nociceptive integration: does it have a peripheral component. *Pain Forum* 1998; 7: 71-78.
- 37 Hassan AH, Ableitner A, Stein C, Herz A. Inflammation of the rat paw enhances axonal transport of opioid receptors in the sciatic nerve and increases their density in the inflamed tissue. *Neuroscience* 1993; 55: 185-195.
- 38 Stein C. The control of pain in peripheral tissue by opioids. *N Engl J Med* 1995; 332: 1685-1690.
- 39 Levine JD, Taiwo YO. Involvement of the mu-opiate receptor in peripheral analgesia. *Neuroscience* 1989; 32: 571-575.
- 40 Goins WF et al. A novel latency active promoter is contained within the herpes simplex virus type 1 UL flanking repeats. *J Virol* 1994; 68: 2239-2252.
- 41 Liu F, Housley PR, Wilson SP. Initial processing of human pro-enkephalin in bovine chromaffin cells. *J Neurochem* 1996; 67: 1457-1462.
- 42 DeLuca NA, McCarthy AM, Schaffer PA. Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4. *J Virol* 1985; 56: 558-570.
- 43 Rasty S, Goins WF, Glorioso JC. Site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 (HSV-1) genome using a cell-free Cre-lox recombination system. In: Adolph KW (ed.). *Methods in Molecular Genetics*, vol 7. Academic Press: London, 1995, pp 114-130.
- 44 Gage PJ, Sauer B, Levine M, Glorioso JC. A cell-free recombination system for site-specific integration of multigenic shuttle plasmids into the herpes simplex virus type 1 genome. *J Virol* 1992; 66: 5509-5515.
- 45 Mestre C et al. A method to perform direct transcutaneous intrathecal injection in rats. *J Pharmacol Toxicol Meth* 1994; 32: 197-200.

## Antihyperalgesic effects of infection with a preproenkephalin-encoding herpes virus

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**ABSTRACT** To test the utility of gene therapeutic approaches for the treatment of pain, a recombinant herpes simplex virus, type 1, has been engineered to contain the cDNA for an opioid peptide precursor, human preproenkephalin, under control of the human cytomegalovirus promoter. This virus and a similar recombinant containing the *Escherichia coli lacZ* gene were applied to the abraded skin of the dorsal hindpaw of mice. After infection, the presence of  $\beta$ -galactosidase in neuronal cell bodies of the relevant spinal ganglia (*lacZ*-containing virus) and of human proenkephalin (preproenkephalin-encoding virus) in the central terminals of these neurons indicated appropriate gene delivery and expression. Baseline foot withdrawal responses to noxious radiant heat mediated by A $\delta$  and C fibers were similar in animals infected with proenkephalin-encoding and  $\beta$ -galactosidase-encoding viruses. Sensitization of the foot withdrawal response after application of capsaicin (C fibers) or dimethyl sulfoxide (A $\delta$  fibers) observed in control animals was reduced or eliminated in animals infected with the proenkephalin-encoding virus for at least 7 weeks postinfection. Hence, preproenkephalin cDNA delivery selectively blocked hyperalgesia without disrupting baseline sensory neurotransmission. This blockade of sensitization was reversed by administration of the opioid antagonist naloxone, apparently acting in the spinal cord. The results demonstrate that the function of sensory neurons can be selectively altered by viral delivery of a transgene. Because hyperalgesic mechanisms may be important in establishing and maintaining neuropathic and other chronic pain states, this approach may be useful for treatment of chronic pain and hyperalgesia in humans.

Information related to noxious thermal, mechanical, and chemical stimuli is transmitted from the skin and other peripheral sites to the central nervous system by myelinated A $\delta$  and unmyelinated C nociceptors (1). Both types of neurons synapse in the dorsal horn of the spinal cord onto various second-order neurons that either carry information to the thalamus and other brain centers or synapse on other spinal neurons. Transmission of sensory information by both types of primary afferents is mediated predominantly by the excitatory amino acid glutamate. Additionally, C fibers appear to release the amino acid aspartate as well as a variety of neuropeptides, including substance P and calcitonin gene-related peptide (1).

In the dorsal horn, application of exogenous opioids such as morphine or release of endogenous opioid peptides, including the enkephalins and dynorphin, activated either by descending or other sensory inputs that activate local circuit neurons, produces analgesia (2, 3). The actions of these endogenous opioid peptides appear to involve activation of  $\mu$ ,  $\delta$  or  $\kappa$  opioid

receptors, all of which are present in the dorsal horn (3, 4). These receptors are predominantly located on the presynaptic terminals of the primary afferents, although postsynaptic opioid receptors are also present (4, 5). These opioid receptors are coupled to G-proteins that inhibit adenylate cyclase, inhibit calcium channels, and stimulate potassium channels, thus tending to reduce neuronal responsiveness (3, 6, 7).

Preproenkephalin A is one of three genes encoding endogenous opioid peptides (8–10). Its product, proenkephalin, is synthesized in a wide variety of central and peripheral neurons, including substantia gelatinosa interneurons of the dorsal horn and in chromaffin cells of the adrenal medulla (11–15). Mice made deficient in preproenkephalin by knockout procedures display several altered responses to painful or threatening environmental stimuli (16). Although these mice do not display altered spinal nociception by the tail-flick assay, their response to the formalin test was altered significantly, closely resembling that of naloxone-treated mice. These findings suggest that endogenous enkephalins do participate in the response to nociceptive stimuli.

Several of the properties of herpes simplex virus type 1 (HSV-1) make it an obvious candidate for gene delivery to the peripheral nervous system (for reviews, see refs. 17–19). First, the virus is neurotropic. Experimental infections in mice show that not only the trigeminal ganglia but also spinal ganglia neurons can be latently infected with human HSV-1 after inoculation of skin or internal organs. Second, in latently infected neurons, the viral DNA is maintained for the life of the host in an episomal form without replication and with little or no expression of viral proteins. Only one region of the genome is actively transcribed during this period, the latency-associated transcript. Third, the large size of the viral genome allows addition of 10–15 kilobases of DNA, adequate for introduction of most genes of interest. Fourth, the viruses are easily manipulated in tissue culture, although the time required to construct and purify a recombinant virus is 3–6 months.

Expression of a variety of marker and other genes encoded by recombinant HSV-1 vectors has been demonstrated *in vivo* (20–22). In cells of the dorsal root and trigeminal ganglia (containing the cell bodies of sensory neurons), expression of transgenes under the control of strong constitutive promoters can persist for months despite little or no viral protein synthesis. This long-term expression has been observed with marker genes under control of the human cytomegalovirus immediate-early enhancer-promoter (22) and the Moloney murine leukemia virus long terminal repeat in combination with one of the two latency-associated transcript promoters, LAP1 (21, 23). The present study uses a recombinant HSV-1 vector to deliver the human preproenkephalin (hPPE) gene to

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sensory neurons. The results demonstrate that proenkephalin is synthesized and processed to active opioid peptides in sensory neurons and that release of these peptides in the spinal cord is antihyperalgesic.

## METHODS

**Virus Construction.** A cassette containing the human cytomegalovirus immediate-early promoter/enhancer, SV40 intron, human preproenkephalin cDNA, and SV40 polyadenylation sequence was cloned from plasmid pCMVhPPE (24) into pBluescript II SK(±) (Stratagene) containing an inserted lox P site. This plasmid was reacted with Cre recombinase in the presence of ICP4<sup>+</sup> viral DNA containing a lox P site in the thymidine kinase locus, as described (25). The virus, designated SHPE, was isolated on a complementing cell line and was purified by three rounds of limiting dilution (26). Virus SHPE was rescued by reintroduction of ICP4 DNA with selection on Vero cells, generating a recombinant vector designated KHPE. Southern blotting confirmed correct insertion of the proenkephalin cassette. KHPE-mediated expression of proenkephalin was verified by infection of primary bovine adrenal chromaffin cells and BSC-40 cells followed by Western blotting with antiproenkephalin mAb PE-25 (27, 28).

**Animal Procedures.** Animal procedures were approved by the University of South Carolina and University of Illinois at Chicago Institutional Animal Care and Use Committees. Female Swiss-Webster mice (5–6 weeks old; Harlan Breeders, Indianapolis) were used for all experiments. Animals were anesthetized with 400 mg/kg of tribromoethanol. The hair was removed from the dorsal surface of each hindfoot, and the skin was scarified by using medium-coarse sandpaper. Five microliters of viral suspension ( $0.5\text{--}1.0 \times 10^8$  plaque-forming units) or vehicle then was applied to one foot and was spread by using the side of a pipettor tip. After ~10 min, when all of the liquid was adsorbed or evaporated, the animals were returned to their home cages. For killing, animals again were anesthetized with tribromoethanol (500 mg/kg) and were perfused by cardiac puncture with PBS followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.5). Dissected spinal cords with ganglia attached were incubated further in fixative for 1–2 h at 4°C.

**Histochemical Procedures.** After washing three times with 0.1 M sodium phosphate (pH 7.5),  $\beta$ -galactosidase activity was visualized in mouse dorsal root ganglia by incubation in this buffer containing 2 mM  $\text{MgCl}_2$ , 5 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 5 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 0.01% sodium deoxycholate, 0.02% Nonidet P-40, and 1 mg/ml X-Gal (stock 20 mg/ml in dimethylformamide) at 37°C for 20–24 h (20). For immunohistochemistry, the lumbar spinal cord was placed in PBS containing 30% sucrose for 24 h and thereafter was blocked in the transverse plane. Cryostat sections (40  $\mu\text{m}$ ) were washed three times in PBS containing 4% normal goat serum and 0.3% Triton X-100, then were incubated for 12 h with gentle agitation at 4°C with a 1:500 dilution of mouse mAb PE-24. Antibody PE-24 binds to amino acids 175–185 of the human preproenkephalin sequence (28) and does not cross-react with either rat or bovine preproenkephalin as bacterially expressed products (B. A. Spruce, personal communication). As a control, other sections were processed in parallel without primary antibody. Sections were incubated further in a 1:50 solution of Texas Red-conjugated goat anti-mouse (The Jackson Laboratory) in PBS for 1 h in the dark (22°C) with constant agitation. The sections then were washed three times with PBS and were incubated with a Met-enkephalin antibody (Peninsula Laboratories; 1:250 solution prepared as for PE-24). After 12 h, the sections were washed three times in PBS and were transferred to a 1:50 solution of sheep anti-rabbit FITC (The Jackson Laboratory) for 1 h in the dark. The sections were washed three times in PBS, were mounted on gelatin-coated slides, were air dried,

were coverslipped with Fluoromount, and were examined under epifluorescence.

**Behavioral Testing.** The foot withdrawal response to noxious radiant heat previously used for rats (29) was adapted for use in mice. Mice were anesthetized lightly with 600 mg/kg urethane and were examined for the time required for foot withdrawal after thermal stimulation of the dorsum of the hindpaw. Animals were tested both at low rates of heating, stimulating predominantly C nociceptors, and high heating rates, mainly stimulating A $\delta$  nociceptors (30, 31). Where indicated, sensitization of C-fiber, low heating-rate responses was achieved by topical application of 20  $\mu\text{l}$  of 0.1% capsaicin (in  $\text{H}_2\text{O}$ /ethanol; 50/50 vol/vol) to the infected foot (30). In other experiments, A $\delta$  nociceptors were sensitized with topical dimethyl sulfoxide (100%), which produces a decrease in response latency for the high but not low rate of foot heating (31). In most experiments, after determining response latencies, either 50 mg/kg naloxone (i.p.), 1.0 mg/kg naloxone (intrathecally), or a comparably delivered vehicle was administered to determine whether endogenous release of opioid peptides mediated any difference observed in response latencies between different groups of animals. Analysis of variance demonstrated that responses of animals not infected with virus were identical to those infected with the KHZ virus in all experiments. Analyses of variance also were performed to determine whether responses of animals infected with the KPE virus were different from those that had been infected with the KHZ virus. Follow-up analyses (Tukey) were performed to determine whether latencies at various time points were significantly different between groups, including after sensitization and after application of naloxone.

## RESULTS

A recombinant HSV-1 vector was constructed to express the human preproenkephalin cDNA under the control of the human cytomegalovirus immediate-early promoter/enhancer (Fig. 1). This virus, designated KHPE, contains the promoter-transgene construct inserted into the viral thymidine kinase gene. This insertion of transgenes into the thymidine kinase gene inactivates the viral gene (32) and disables viral replication in nondividing cells such as neurons (33). A similar recombinant virus, designated KHZ, containing the *E. coli lacZ* gene in place of the human preproenkephalin cDNA also was used (34, 35). Expression of  $\beta$ -galactosidase in sensory neurons of mouse dorsal root ganglia was examined after infecting animals on the dorsal skin of the hindfoot with KHZ. At 4 days postinfection, KHZ-infected animals exhibited histochemical reactions for  $\beta$ -galactosidase in 20–100 cell bodies in the L3–L5 ganglia ipsilateral to the infection (Fig. 2B), with occasional staining in the L2 ganglion. Similar levels of expression were observed at 2 weeks postinfection (results not shown). At 5–6 weeks postinfection, both the number and intensity of staining for  $\beta$ -galactosidase was reduced in KHZ-infected animals (Fig. 2C), indicative of reduced enzyme expression, between 10 and 25% of that observed at 4 days.

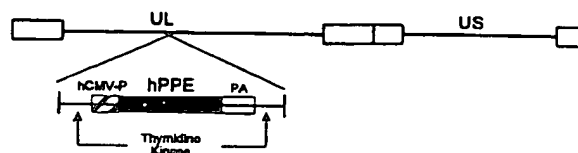


Fig. 1. Schematic representation of recombinant herpes virus KHPE. See *Methods* for virus construction. UL, unique long segment; US, unique short segment; hCMV-P, human cytomegalovirus immediate-early promoter/enhancer; hPPE, human preproenkephalin cDNA; PA, simian virus 40 polyadenylation sequence.

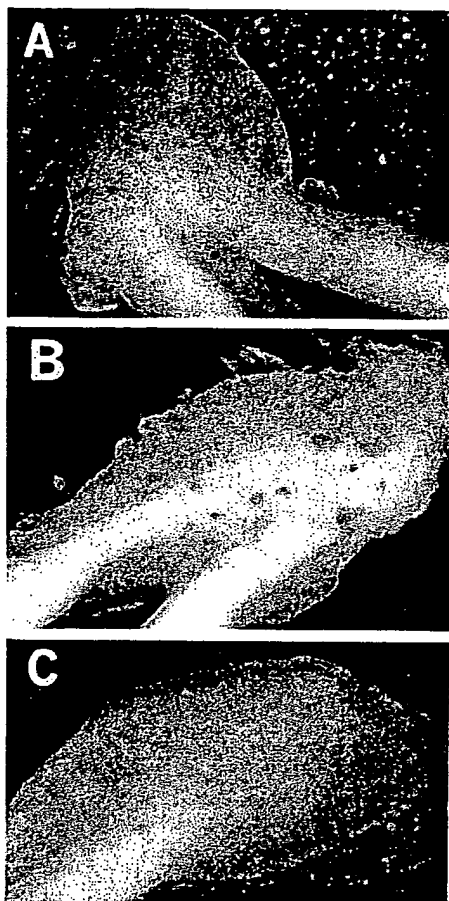


FIG. 2. Histochemical staining for HSV-1 vector-mediated  $\beta$ -galactosidase expression in mouse dorsal root ganglia. Swiss-Webster mice (6 weeks old) were infected, where indicated, with  $5 \times 10^7$  plaque-forming units of virus. Staining in the ipsilateral L3 ganglia is shown. (A) Animal with no virus infection. (B) Animal infected with virus KHZ and killed 4 days postinfection. (C) Animal infected with virus KHZ and killed 40 days postinfection.

Expression of  $\beta$ -galactosidase at 10 weeks postinfection was similar to that at 5–6 weeks (results not shown).

Expression of human proenkephalin in the mouse spinal dorsal horn and axons of sensory neurons in nerve roots after infection with virus KHPE was detected by using a human-specific mAb directed against proenkephalin (Fig. 3A–C). Human proenkephalin immunoreactivity was observed in primary afferent axons in dorsal roots (Fig. 3A), in axons invading the dorsal horn (Fig. 3B), and in primary afferent terminals in the substantia gelatinosa of the dorsal horn (Fig. 3C). In all cases, human proenkephalin immunoreactivity was restricted to the side ipsilateral to the infection. Some of the proenkephalin terminals were also immunoreactive for the fully processed peptide neurotransmitter Met-enkephalin (Fig. 3D).

Animals infected with recombinant viruses KHZ and KHPE also were evaluated for effects on foot withdrawal responses evoked by noxious radiant heat in normal and hyperalgesic mice. This method uses different rates of noxious skin heating, which allows separate assessment of behavioral responses produced by the activation and sensitization of myelinated (A $\delta$ ) or unmyelinated (C) nociceptors. Specifically, high-rate heating (6.5°C/min) and topical dimethyl sulfoxide preferentially activate and selectively sensitize A $\delta$  nociceptors whereas low-rate heating (0.9°C/min) and topical capsaicin selectively activate and sensitize C fiber nociceptors (29–31, 36). Animals were infected with virus on the depilated and scarified dorsal

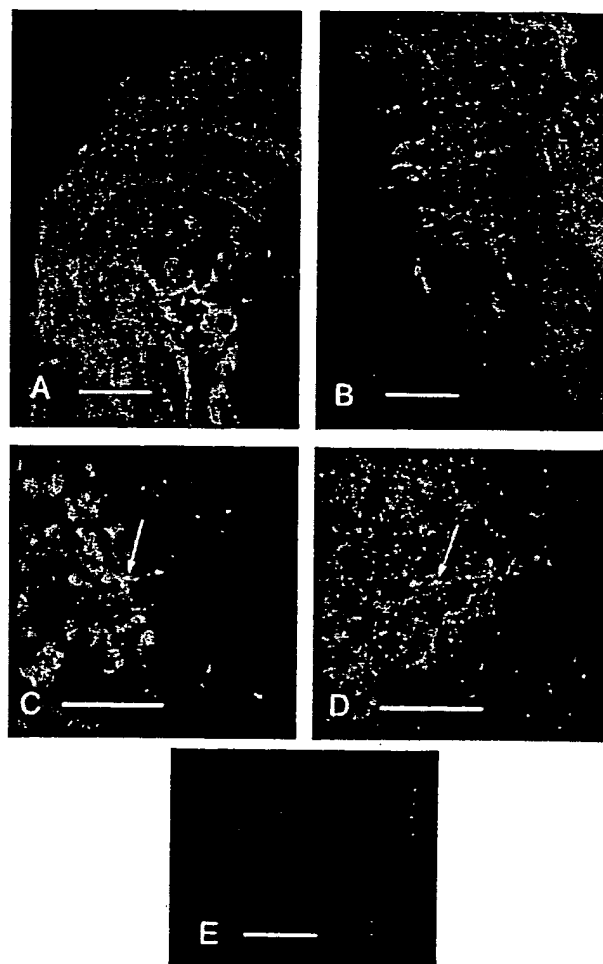


FIG. 3. Immunohistochemistry of HSV-1 vector-mediated expression of human proenkephalin in mouse dorsal roots and spinal cord. (A) Human proenkephalin (PE-24) immunoreactivity in dorsal root axons of a left dorsal rootlet visualized with Texas Red. Dorsal is down, medial (toward cord) is to the right. (Bar = 100  $\mu$ m.) (B) Human proenkephalin immunofluorescence in primary afferent axons at the dorsal root entry zone of the dorsal horn ipsilateral to the original infection. Dorsal is up, medial is to the left, and the edge of spinal cord is to the right. (Bar = 100  $\mu$ m.) (C) Human proenkephalin immunofluorescence in primary afferent terminals (arrow) in the inner substantia gelatinosa. Dorsal is up, and medial is to the right. (Bar = 20  $\mu$ m.) (D) Met-enkephalin immunoreactivity in the same afferent terminals as in C (arrow) visualized with fluorescein. Area is chosen for relative paucity of Met-enkephalinergic terminals. (Bar = 20  $\mu$ m.) (E) Lack of human proenkephalin (PE-24) immunoreactivity in dorsal horn at right dorsal root entry zone contralateral to the original infection visualized with Texas Red. Dorsal is up, and the outside of the cord is to the right. (Bar = 100  $\mu$ m.)

skin of the hindfoot and were tested for foot withdrawal latency to both high and low rates of heating between 1 and 6 weeks postinfection. Results from this testing are shown in Fig. 4. Baseline latencies for both rates of heating were similar for animals infected with KHPE or KHZ and were similar to latencies recorded for uninfected animals. In uninfected mice and in mice infected with KHZ, application of capsaicin sensitized C nociceptors (Fig. 4A and B) but not A $\delta$  nociceptors (results not shown), as evidenced by significantly ( $P < 0.05$ , ANOVA) reduced foot withdrawal latencies (30). In contrast, mice infected with KHPE exhibited no such sensitization ( $P > 0.05$ , ANOVA). In a similar manner, the selective reduction of A $\delta$  nociceptor-mediated responses induced by application of dimethyl sulfoxide was blocked by infection with

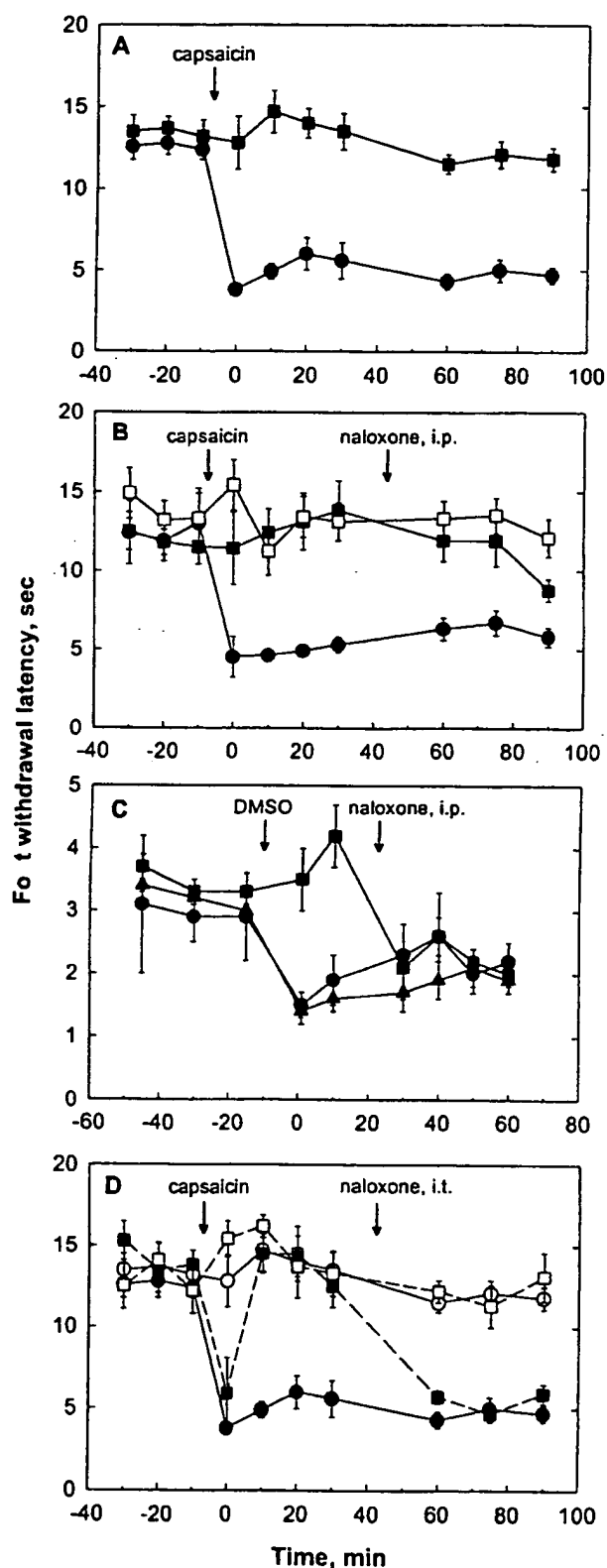


FIG. 4. Foot withdrawal responses to noxious radiant heat. Animals were infected unilaterally with either KHZ or KHPE. Topical capsaicin (A, B, and C) or DMSO (C) was applied to the infected foot as indicated. Data are presented as mean  $\pm$  SEM,  $n = 6-8$ . (A) Response to a low rate of skin heating, preferentially activating C nociceptors.  $\bullet$ , KHZ-infected animal, ipsilateral foot;  $\blacksquare$ , KHPE-infected animal, ipsilateral foot. (B) Response to a low rate of skin heating. Naloxone (50 mg/kg i.p.) was administered where indicated.

KHPE (Fig. 4C), as evidenced by the significant difference ( $P < 0.05$ , ANOVA) between KHPE-infected and KHZ-infected animals. This blockade of both A $\delta$  and C fiber-mediated hyperalgesia in KHPE-infected animals was observed at all times examined, although it appeared more robust at 4 and 6 weeks than at 1–2 weeks after infection (results not shown). In some cases, a reversal of sensitization appeared only after an initial hyperalgesic response (Fig. 4D).

Systemic administration of the opioid antagonist naloxone (50 mg/kg, i.p.) partially (C-fiber response) or completely (A $\delta$ -fiber response) restored sensitization in KHPE-infected animals but had no effect on KHZ-infected or mock-infected animals (Fig. 4B and C), indicating at least partial opioid mediation of the observed antihyperalgesic effects. Intrathecal administration of naloxone (1.0 mg/kg) also completely reversed the KHPE-induced blockade of sensitization for C fiber-mediated responses (Fig. 4D), indicating that at least part of the antihyperalgesic effect of the virus was mediated by spinal release of opioids. Naloxone administration in KHPE-infected or KHZ-infected animals without prior sensitization was without effect (not shown).

## DISCUSSION

The present results demonstrate prolonged, herpes-mediated expression of transgenes in mouse sensory neurons. Although expression of  $\beta$ -galactosidase at 5–6 weeks postinfection was reduced to levels 10–25% of those observed at 4 days, expression was observed without further diminution up to 10 weeks, the longest time tested. A similar time course of transgene expression has been reported by others (22). The finding that the human cytomegalovirus immediate-early promoter/enhancer drives expression of transgenes, both acutely and after establishment of latency, confirms previous studies (22) on the utility of this promoter for expression in sensory neurons. Although a number of other promoters have been tested for acute and prolonged expression in the murine sensory neurons, the only other promoter demonstrated to maintain transgene expression during viral latency *in vivo* is the Moloney murine leukemia virus long terminal repeat (20, 21, 23).

The results also demonstrate that infection of peripheral endings of sensory neurons with recombinant HSV-1 encoding a neuropeptide precursor, human proenkephalin, results in synthesis and processing of this precursor in mouse sensory neurons. A similar viral construct that results in opioid peptide synthesis in primary afferents was reported while this work was in progress (37). The presence of both the precursor and processed enkephalins in central terminals of the primary afferents (Fig. 3) shows production of biologically active transgene products (8–15). The results further demonstrate that infection of cutaneous nociceptive afferents with a proenkephalin-encoding HSV-1 produces opioid-mediated antihyperalgesia without affecting basal nociceptive responses. This finding has been confirmed with two additional viral recombinants encoding proenkephalin (S.P.W. and D.C.Y., unpublished work). In contrast, animals that received infections of a *lacZ*-containing HSV-1 did not show any changes in their nociceptive responsiveness. These data clearly suggest that

$\bullet$ , KHZ-infected animal, ipsilateral foot;  $\blacksquare$ , KHPE-infected animal, ipsilateral foot;  $\square$ , KHPE-infected animal, contralateral foot (no capsaicin applied). (C) Response to a high rate of skin heating, preferentially activating A $\delta$  nociceptors.  $\bullet$ , KHZ-infected animal, ipsilateral foot;  $\blacksquare$ , KHPE-infected animal, ipsilateral foot;  $\triangle$ , uninfected animal. (D) Response to a low rate of skin heating. Naloxone (1 mg/kg intrathecally) was administered where indicated. KHZ-infected animal (solid line):  $\bullet$ , ipsilateral foot;  $\circ$ , contralateral foot (no capsaicin applied). KHPE-infected animal (broken line):  $\blacksquare$ , ipsilateral foot;  $\square$ , contralateral foot (no capsaicin applied).

viral introduction of the human proenkephalin cDNA into nociceptive primary afferents alters the responsiveness of both C and A $\delta$  afferents to stimuli which would normally produce hyperalgesia.

The mechanisms by which the observed antihyperalgesia occurs are not clear however. Blockade of the effects of the proenkephalin-encoding virus by intrathecal naloxone suggests that observed antihyperalgesic effect is mediated by the release of opioid peptides from the central terminals of the primary afferents, although involvement of peripheral opioid receptors cannot be excluded (38). In this way, the observed opioid-mediated antihyperalgesia mimics the well known effects of endogenous or intrathecally administered enkephalins (2–4). However, the lack of effect on basal foot withdrawal responses suggests that the opioids effecting the antihyperalgesia may not be tonically released in the infected animals but, rather, may be released only when there is a substantial activation of the afferents. In support of this is the finding that, in KHPE-infected animals that were not exposed to capsaicin or DMSO, naloxone did not alter response latencies as would be expected if opioids were being released tonically. It is also interesting to note that the dosages of naloxone given were quite high, possibly indicating a nonspecific effect. Preliminary experiments indicated that such high doses were necessary to reverse the antihyperalgesia. If enkephalins were being released very near to presynaptic terminals, high doses of antagonists might be necessary to block the locally high concentrations of the peptides.

Several issues raised by these studies will require further study. For example, the specific site, presynaptic versus postsynaptic, at which the opioid peptides act is unclear. Presynaptic opioid effects have been demonstrated for both A $\delta$  and C fiber-mediated responses (see refs. 5 and 6). Taken together with these previous findings of presynaptic opioid effects, the results are consistent with at least part of the antihyperalgesic effects resulting from autoinhibition of nociceptive afferents at the presynaptic terminals. A second question raised is that of the development of opioid receptor tolerance. Although our results suggest that enkephalin release depends on afferent activity, development of tolerance could be problematic during long periods of nociception. We are currently investigating this possibility.

An issue raised by these results is the apparent lack of a direct correlation between marker transgene expression and the behavioral effects. Expression of  $\beta$ -galactosidase in the dorsal root ganglia of KHZ-infected rats decreases by at least 75% from 4 days to 6 weeks postinfection. Although the antihyperalgesic effects of KHPE were observed as soon as 4–5 days postinfection, the reproducibility and magnitude of these effects appeared to increase over at least the first 2 weeks and remained robust until at least 7 weeks postinfection (the longest time tested). Several factors could account for this apparent discrepancy including (i) the time required for complete healing of the viral inoculation site, (ii) the time required to synthesize and transport an adequate number of vesicles containing proenkephalin products to the nerve terminals, (iii) modulation of sensory function by very low levels of proenkephalin products, or (iv) proenkephalin-induced plastic changes in the sensory neurons. Further studies will be required to examine possible plastic changes. Even the lower levels of transgene expression observed (Figs. 2 and 3) at 4–6 weeks may be adequate to maintain peptide stores in these neurons in which turnover may be low in the absence of robust stimuli. If future work demonstrates that the antinociceptive effect also diminishes over greater time periods than that tested, it will be of interest to determine whether reinfection will demonstrate effects similar to the original infection.

The results of the present study are exciting because they imply that, by viral delivery of a transgene, the function of sensory neurons can be selectively altered. In this case, hyper-

algesia was blocked without altering baseline nociception. If the neuropeptide precursor proenkephalin is being synthesized, processed to active peptides, and stored in secretory vesicles in the infected sensory neurons, as the present studies seem to suggest, a pool of releasable enkephalins will exist in the terminals of these neurons, possibly stored with other nociceptive neuropeptides, including substance P and calcitonin gene-related peptide. Basal release of the transgene products should be minimal; vesicular storage, probably in large dense-core vesicles, would limit enkephalin release to those instances of high-level or persistent stimulation necessary to evoke peptide release.

These observations further suggest that KHPE or a similar recombinant herpes virus may be useful for treatment of chronic pain in humans. Hyperalgesia, which may be important in establishing and maintaining neuropathic and other chronic pain states, was selectively blocked by infection with this proenkephalin-encoding virus. Advantages of this type of gene therapy would include precise anatomical targeting of the specific nociceptors transmitting pain impulses, the lack of systemic opioid adverse effects, and a long (weeks to months) duration of action. In addition, it appears that normal sensory transmission would not be disrupted when persistent and robust pain sensitization are absent.

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1. Bonica, J. J. (1990) *The Management of Pain* (Lea and Febiger, Philadelphia).
2. Yaksh, T. L. & Noueihed, R. (1985) *Annu. Rev. Pharmacol. Toxicol.* 25, 433–462.
3. Mansour, A., Fox, C. A., Akil, H. & Watson, S. J. (1994) *Trends Neurosci.* 18, 22–29.
4. Ji, R. R., Zhang, Q., Law, P. Y., Low, H. H., Elde, R. & Hökfelt, T. (1995) *J. Neurosci.* 15, 8156–8166.
5. Lu, Y., Pirec, V. & Yeomans, D. C. (1997) *Br. J. Pharmacol.* 121, 1210–1216.
6. Glaum, S. R., Miller, R. J. & Hammond, D. L. (1994) *J. Neurosci.* 14, 4965–4971.
7. Werz, M. A. & MacDonald, R. L. (1982) *Brain Res.* 239, 315–321.
8. Comb, M., Seeburg, P. H., Adelman, J., Eiden, L. & Herbert, E. (1982) *Nature (London)* 295, 663–666.
9. Gubler, U., Seeburg, P. H., Hoffman, B. J., Gage, P. & Udenfriend, S. (1982) *Nature (London)* 295, 206–208.
10. Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hirose, T., Inayama, S., Nakanishi, S. & Numa, S. (1982) *Nature (London)* 295, 202–206.
11. Yang, H.-Y. T., DiGiulio, A. M., Fratta, W., Hong, J. S., Majane, E. A. & Costa, E. (1980) *Neuropharmacology* 19, 209–215.
12. Mucchetti, I., Guidotti, A., Schwartz, J. P. & Costa, E. (1985) *J. Neurosci.* 5, 3379–3385.
13. Yang, H. Y., Hong, J. S. & Costa, E. (1977) *Neuropharmacology* 16, 303–307.
14. Hökfelt, T., Ljungdahl, A., Terenius, L., Elde, R. & Nilsson, G. (1977) *Proc. Natl. Acad. Sci. USA* 74, 3081–3085.
15. Sar, M., Stumpf, W. E., Miller, R. J., Chang, K. J. & Cuatrecasas, P. (1978) *J. Comp. Neurol.* 182, 17–37.
16. König, M., Zimmer, A. M., Steiner, H., Holmes, P. V., Crawley, J. N., Brownstein, M. J. & Zimmer, A. (1996) *Nature (London)* 383, 535–538.
17. Fink, D. J., DeLuca, N. A., Goins, W. F. & Glorioso, J. C. (1996) *Annu. Rev. Neurosci.* 19, 265–287.
18. Smith, F., Jacoby, D. & Breakfield, X. O. (1995) *Restor. Neurol. Neurosci.* 8, 21–34.
19. Glorioso, J. C., DeLuca, N. A. & Fink, D. J. (1995) *Annu. Rev. Microbiol.* 49, 675–710.
20. Dobson, A. T., Margolis, T. P., Sedarati, F., Stevens, J. G. & Feldman, L. T. (1990) *Neuron* 5, 353–360.

21. Lokensgard, J. R., Bloom, D. C., Dobson, A. T. & Feldman, L. T. (1994) *J. Virol.* 68, 7148–7158.
22. Ecob-Prince, M. S., Hassan, K., Denheen, M. T. & Preston, C. M. (1995) *J. Gen. Virol.* 76, 1527–1532.
23. Davar, G., Kramer, M. F., Garber, D., Roca, A. L., Andersen, J. K., Bebrin, W., Coen, D. M., Kosz-Vnenchak, M., Knipe, D. M., Breakfield, X. O., *et al.* (1994) *J. Comp. Neurol.* 339, 3–11.
24. Liu, F., Housley, P. R. & Wilson, S. P. (1996) *J. Neurochem.* 67, 1457–1462.
25. Gage, P. J., Sauer, B., Levine, M. & Glorioso, J. C. (1992) *J. Virol.* 66, 5509–5515.
26. Kang, W., Wilson, M. A., Bender, M. A., Glorioso, J. C. & Wilson, S. P. (1998) *Brain Res.* 792, 133–135.
27. Spruce, B. A., Curtis, R., Wilkin, G. P. & Glover, D. M. (1990) *EMBO J.* 9, 1787–1795.
28. Böttger, V., Böttger, A., Lane, E. B. & Spruce, B. A. (1995) *J. Mol. Biol.* 247, 932–946.
29. Yeomans, D. C. & Proudfit, H. K. (1994) *Pain* 59, 85–94.
30. Yeomans, D. C. & Proudfit, H. K. (1996) *Pain* 68, 141–150.
31. Yeomans, D. C., Pirec, V. & Proudfit, H. K. (1996) *Pain* 68, 133–140.
32. Krisky, D. M., Wolfe, D., Marconi, P. C., Ramakrishnan, R., Mata, M., Rouse, R. J. D., Fink, D. J. & Glorioso, J. C. (1998) *Gene Ther.* 5, 1593–1603.
33. Tenser, R. B., Hay, K. A. & Erdis, W. A. (1989) *J. Virol.* 63, 2861–2865.
34. Mester, J. C., Pitha, P. M. & Glorioso, J. C. (1995) *Gene Ther.* 2, 187–196.
35. Rasty, S., Goins, W. F. & Glorioso, J. C. (1995) *Methods Mol. Genet.* 7, 114–129.
36. Baumann, T. K., Simone, D. A., Shain, C. N. & LaMotte, R. H. (1991) *J. Neurophysiol.* 66, 212–227.
37. Bras, J. M. A., Epstein, A. L., Bourgoin, S., Hamon, M., Cesselin, F. & Pohl, M. (1998) *J. Neurochem.* 70, 1299–1303.
38. Kinnman, E., Nygards, E. B. & Hansson, P. (1997) *Anesth. Analg.* 84, 595–599.